Range-wide chloroplast DNA phylogeographies of three widespread Australian cool temperate rainforest plants

by

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

University of Tasmania (November, 2009)

Declarations

This thesis does not contain any material which has been accepted for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgment is made in the text of the thesis.

James R. P. Worth

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Abstract

How temperate plants and animals survived hostile climates during past glacial periods is critical to understanding modern ecological communities. Species may have survived in macro-refugia, and migrated large distances to reach their current range since climates recovered 10-12,000 years ago, or may have survived in many micro-refugia. The cool temperate rainforest of southeastern Australia provides opportunities to better understand how these processes have shaped the current biota. This thesis investigates the range-wide chloroplast DNA phylogeographies of three widespread Australian cool temperate rainforest plants.

In the gravity-dispersed tree, *Nothofagus cunninghamii* (Nothofagaceae), 23 haplotypes were identified by PCR-RFLP and sequencing of 2164 base pairs of chloroplast DNA from 342 individuals. Deep haplotype divergence occurred, with the haplotype of *N*. *moorei* nested among those of *N. cunninghamii*. Western Tasmania was the stronghold of haplotype diversity, with evidence for multiple glacial refugia in coastal and inland locations. Three haplotypes endemic to the Victorian Central Highlands corroborate pollen evidence for last glacial maximum survival. In eastern Tasmania, an endemic and deeply diverged haplotype suggests long-term occupation within this region.

In the bird-dispersed shrub *Tasmannia lanceolata* (Winteraceae) 30 haplotypes were identified by sequencing of 3190 base pairs of chloroplast DNA from 244 individuals. Strong phylogeographic structuring, with eight clades with predominantly non-overlapping geographic distributions, provided evidence for multiple glacial refugia, including within the driest parts of the species' range. This strong structure may be the result of the lack of vectors for effective long-distance dispersal, such as migratory birds. However, other factors limiting establishment at long distances, such as dioecy, competition, or selection may have contributed.

Sequences of 3295 base pairs from 142 samples of the wind-dispersed tree, *Atherosperma moschatum* (Atherospermataceae), revealed low chloroplast variation (six haplotypes). The apparently ancestral haplotype was widespread across Tasmania. A single haplotype was found across all Victorian populations, while the most diverged haplotypes were observed near the northern limit of the species' range in New South Wales.

The deep phylogeographic patterns in *N. cunninghamii* and *T. lanceolata* are the result of probable long-term survival in multiple regions, indicating the resilience of these species in apparently hostile last glacial climates. Differing dispersal traits have not affected the limited mobility of these species in response to past climatic changes. This contrasts with the extensive continent wide migrations of temperate species in the northern hemisphere. However, the very low diversity in *A. moschatum* may indicate a divergent history of this species.

Acknowledgements

I would like to thanks my supervisors Greg Jordan and René Vaillancourt who have been so patient and supportive throughout this work. I would like to thank them both for teaching me so much, and there useful insights that improved this project. I would also like to thank my research supervisor Gay McKinnon for assistance with phylogenetic techniques.

This project would have taken far longer without some help in the field and in the lab. I would specifically like to thank Adam Smolenski for his fantastic help in the lab when things weren't working and James Marthick who was gave invaluable assistance in the lab and field and showed great enthusiasm for this project. Many people helped me out by collecting samples including J and N Cross (King Island), Greg Dutkowski, Taylor Feild, Dave Handscombe (Parks Vic), Corey Hudson, Jasmine Janes, Sue Jennings, Rebecca Jones, Anthony Mann, Jon Marsden-Smedley, Trevor McIntosh (Parks Vic), Simon Whittock and Dave Woods (Parks NSW). I also acknowledge the useful advice and discussions I had at times with Tim Brodribb, Bob Elliot, Des Fitzgerald, Jules Freeman, Robert Mesibov and Dorothy Steane.

I would like to thank my parents who were gave me a place to live throughout most of this work and Ray Worth was a great help during field trips in Tasmania. Lastly, I would like to thank Madoka for her support, patience and encouragement while I undertook this work.

Publications from this thesis

Refereed articles

Worth, JRP, Jordan, GJ, McKinnon, GE and Vaillancourt, RE. 2009. The major Australian cool temperate rainforest tree *Nothofagus cunninghamii* withstood Pleistocene glacial aridity within multiple regions: evidence from the chloroplast. *New Phytologist* **182**: 519-532.

Conference articles

Worth, JRP, Vaillancourt, RE, Jordan, GJ, McKinnon, GE. 2008. Chloroplast DNA reveals genetic legacy of ice ages in the rainforest species *Nothofagus cunninghamii*. In Proceedings of 'Old Forests, New Management' Sir Mark Oliphant Conference, 17-21 February 2008, Hobart, Australia. p. 67.

Worth, JRP, Vaillancourt, RE, Jordan, GJ. 2004. Where did *Nothofagus cunninghamii* survive the ice ages? In Proceedings of 'Australasian Quaternary Association', 2004, Cradle Mountain, Australia. p. 43.

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Chapter 1: Introduction

General introduction

The first complete images of the Earth taken on the manned flights to the moon 40 years ago showed the blue oceans, the white ice of the poles and the continents covered in vast expanses of green vegetation juxtaposed against wide brown deserts. Rather than being stable, the vegetation of Earth has changed markedly through time. The major biomes have contracted and expanded repeatedly in response to past and ongoing changes in the Earth's climate. On long time-scales the climate of the Earth changes as a consequence of the drifting of the continents and subsequent mountain building and changes in the ocean currents. On a shorter time-scale dramatic changes in global temperature and ice cover have occurred within the last 2.5 million years with repeated and rapid shifts from cold glacial to warm interglacial climates. These warm and cold periods are believed to be caused by changes in the orbit of the earth around the sun (Hays *et al.*, 1976) that result in variations in the amount of solar radiation received by different parts of the earth in different seasons (Bennett, 1990), an effect that is magnified by associated changes in carbon dioxide concentrations of the atmosphere (Kerr, 1984; Pisias & Shackleton, 1984). In the northern hemisphere, summer temperatures were 15- 20°C lower during the maximum of last glacial climates 18,000 years ago than the present interglacial and ice sheets up to 3 km thick covered ~ 50% of the continents of Eurasia and North America (Dawson, 1992). Apart from expansion of the ice sheet on the Antarctic continent, there were only minor glaciations in the southern hemisphere (Lewis & Illgner, 2001; Barrows *et al.*, 2002; Hulton *et al.*, 2002), though there were large expanses of periglacial conditions. In the temperate zone, the fossil pollen record demonstrates that forest species were far less abundant and steppe species dominated during the cold conditions of glacial periods (Tzedakis, 1993). However, with the rapid onset of warmer interglacials forest species recovered markedly in abundance in the fossil record. Of particular importance is the fact that only 12-14,000 years has elapsed since the main phase of climatic recovery, meaning that some forest tree species may have had fewer than 100 generations to regain their ranges.

How plants respond to climatic change, and in particular how forest species have been able to recover so rapidly in space and time at the end of glacials, has long

intrigued biologists. Individual species are generally thought to have climatic envelopes within which they can occur (e.g. bound by temperature, rainfall etc.) that are related to the type and variability of morphological and physiological functional traits of each species (e.g. plant traits that allow species to withstand drought). Because it often exposes species to suboptimal conditions, significant climate change induces three kinds of biotic responses. The first is mobility, involving the movement of individuals principally by seed dispersal into suitable habitats, that is, the tracking of suitable climates. The second is adaptation, which involves the selection of individuals that possess functional traits that allow the species to survive changing climates *in situ*. These may be garnered by the expression of genes that previously were present in the species or population, evolution of new gene variants, or possibly via hybridisation. The third is not strictly a response, instead it represents a lack of response - the toleration of change without significant shifts in range or adaptation. This may involve persistence through unfavourable climates via long lived life stages that include vegetative reproduction enabling survival of individuals without completion of the whole lifecycle (Eriksson, 2000). However, all three responses are not mutually exclusive. If species are unable to move, adapt or tolerate changing climates, they become extinct regionally or globally. The vast majority of all animal and plant species that have ever existed are considered to have gone extinct. However, although there is evidence of extensive extinction in at least some parts of the world during the early Pleistocene glacial cycles e.g. (Jordan, 1995a, b, 1997, 1999), very few global extinctions of plant species have been documented in the late Pleistocene e.g. (Jordan & Hill, 1991; Jackson & Weng, 1999), though a number of regional extinctions are known, for example, Loranthaceae and *Quintinia* from Tasmania; and *Zelkova* in central Italy (Follieri *et al.*, 1986). As a result, it appears that after culling by the first glacial cycles, most temperate species persisted through some combination of range alteration (i.e. mobility), adaptation and/or tolerance.

The fossil record

The fossil record has been one of the traditional sources of evidence on the response of plants to past climatic changes. Most of this evidence comes from palynology (the study of pollen and spores; or palynomorphs), because pollen grains are produced in astronomic numbers, are mobile and are often well preserved in sediment through their durable coat of sporopollenin. In some circumstances unbroken palynological records can be obtained that span tens of thousands of years that give a continuous

representation of past vegetation cover through time. The investigation of multiple palynological sites across landscapes has been used to infer the movement of vegetation types or particular species. For example, extensive palynological records in Europe and North America indicates a wave-like increases in pollen abundance of some key temperate forest taxa northwards at the end of the last glacial from areas of full glacial survival in southern regions (Davis, 1983; Birks, 1989; Magri, 2008). However, fossil palynological data can be unreliable for detecting the presence of plants present at low densities (McLachlan & Clark, 2004). In addition, pollen and spore morphology is often similar between closely related species, so that the palynological record typically documents changes in abundance of genera or families. Macrofossils, that is, fossilized macroscopic remains such as leaves and wood, can provide more certainty for the local presence of plant taxa because they are rarely transported far in contrast to pollen (Rowell *et al.*, 2001). In addition macrofossils can more easily be classified to species, though such fossils are rarer than fossilized pollen.

The molecular approach

Over the last three decades, phylogeography has become a pivotal tool for understanding the distribution of species and ecosystems (Riddle, 2009). Phylogeography analyses the relationships between the geographical distributions of genotypes within and between populations together with the phylogenetic relationships among genotypes (Avise, 1987) with the aim of reconstructing past history of natural populations. Analysis of chloroplast DNA has become the favoured tool for plant phylogeography for a number of reasons. Firstly, the chloroplast genome can contain significant levels of intraspecific diversity, as was shown by some early studies (e.g. Harris & Ingram, 1991; Soltis *et al.*, 1992; Vaillancourt & Weeden, 1992). Secondly, chloroplast DNA is maternally inherited in most angiosperms (Corriveau & Coleman, 1988; Mogensen, 1996) and therefore dispersed by seed which is often less mobile than the other vector for geographic movement of genes, pollen. Thirdly, unlike the nuclear genome, the near absence of recombination and heterozygosity of the chloroplast means that the chloroplast can be regarded as a single haploid gene that is more prone to drift. This can lead to the greater retention of genetic imprints of past migration and isolation of populations than nuclear genes (Ennos *et al.*, 1999). Lastly, the chloroplast is slowly evolving (Wolfe *et al.*, 1987;

Zurawski & Clegg, 1987) which means that past events are unlikely to be erased by new mutations.

Chloroplast phylogeographic studies have generally agreed with locations of glacial refugia suggested by palynology of northern hemisphere temperate biota e.g. (Petit *et al.*, 2003), but have also identified refugia in new locations (Comes & Kadereit, 1998). Importantly, chloroplast information is able to provide information on the limits of spread from separate refugia (Matayas & Sperisen, 2001; Magri *et al.*, 2006), and enable some understanding of the number of individual colonization events involved during establishment of new populations. Areas of high diversity of related haplotypes are generally considered to be signals of long term occupation (e.g. Cannon & Manos, 2003; Jiminez *et al.*, 2004; Fineschi *et al.*, 2005a). In contrast recently colonized areas, especially those far from refugia, are expected to harbour only a subset of the overall genetic diversity present in refugia (Hewitt, 1996) as expansion from refugia is thought to involve few individuals from the fringes of refugia (Taberlet *et al.*, 1998). Where fronts of different migration routes come into contact, admixture zones containing unrelated haplotypes can be observed (Petit *et al.*, 2003; Walter & Epperson, 2005). Recent studies have observed genetic patterns across the ranges of some species that have been inferred as being the genetic imprint of processes that acted long before the last glacial (Lumaret *et al.*, 2002; Grivet *et al.*, 2006; Magri *et al.*, 2007; Morris *et al.*, 2008).

Problem Statement

Understanding the response of plants to climatic change is critical to knowing how plant species will respond under future climate change and the fragmentation of species ranges by humans. Studying how plants have responded to past climatic changes, notably the sometimes rapid changes of the glacial-interglacial cycles, provides a means of informing this debate. However, there is considerable uncertainty about how plants responded to these past changes, centred on the relative importance of mobility and resilience by adaptation and/ or tolerance.

Perhaps the most discussed case of mobility of terrestrial organisms is how plants and animals in the temperate zones were able to recover from the Last Glacial Maximum to establish their present sometimes continent-wide ranges. Fossil pollen evidence from the last glacial to present, particularly from the northern hemisphere, has been used to infer that species were capable of continent wide migrations over

thousands of kilometres from regions that escaped the major effects of glacial climates below the ice sheets and widespread periglacial conditions (Davis, 1983; Huntley & Birks, 1983). Such migration would have needed to have been very rapid, with movements of up 1.5 km per year for some plants (Huntley & Birks, 1983). In most cases, these rates far exceed the modern observations of dispersal capacity in the relevant species. This paradox (Reids paradox; Clark *et al.*, 1998) has been theorised to have been made possible by long distance dispersal allowing species to establish distant founding populations (Cain *et al.*, 1998; Clark *et al.*, 1998; Cain *et al.*, 2000). The frequency of these long distance dispersal events were possibly not affected by the different dispersal types (e.g. bird, animal, wind and gravity) possessed by plants but involved processes of dispersal not related to the morphology of the plant propagule e.g. (Wilkinson, 1997; Jordan, 2001; Higgins *et al.*, 2003).

However, there remains considerable uncertainty about this pollen-based model of rapid dispersal of temperate species after the last glacial. Firstly, there is some doubt concerning the use of pollen records to reconstruct migration rates (MacDonald, 1993). In addition, there is accumulating macrofossil and genetic based evidence for persistence of species close to the ice sheets of Europe and North America, rather than in distant southern refugia (Stewart & Lister, 2001; McLachlan *et al.*, 2005). As a result a number of authors have argued for a greater importance of adaptation/tolerance in the glacial recovery of species. Secondly, there is still poor understanding of the consequences of different dispersal mechanisms for dispersal and therefore the ability of species with differing dispersal traits to shift ranges during climatic changes. Many examples of the genetic structure of the chloroplast DNA in current populations of temperate plants indicates that species with dispersal via wind or animals (especially birds) have less strong structuring than gravity dispersed plants (Raspe *et al.*, 2000; Oddou-Muratorio *et al.*, 2001; Salvini *et al.*, 2001; Mohanty *et al.*, 2001 2002; Palme *et al.*, 2003a; Palme *et al.*, 2003b; Fineschi *et al.*, 2005b; Duminil *et al.*, 2007; Maliouchenko *et al.*, 2007).

Cool temperate rainforest of southeastern Australia- a model system

Cool temperate rainforest is a type of evergreen closed canopy forest (Busby & Brown, 1994) that is widespread in southeastern Australia from 28° S in southern Queensland to 43° S on the island of Tasmania. This biome occurs in high rainfall

areas along the eastern highlands where rainfall exceeds 1500 mm per year but also occurs in some areas receiving down to 750 mm in favourable situations (Webb & Tracey, 1981). Cool temperate rainforest comprises 30% of the approximately 2 million hectares of rainforest in Australia (Webb & Tracey, 1981).

Cool temperate rainforest is distinct from other Australian rainforest types, the warm temperate, subtropical and tropical rainforests (Webb, 1959). The major canopy dominant of cool temperate rainforest differs with latitude. *Nothofagus cunninghamii* (Nothofagaceae, subgenus *Lophozonia*) occurs from southern Tasmania (43.5 °S) to the Central Highlands of Victoria (37 °S) (Figs. 1, 2 and 3). *Eucryphia moorei* (Cunoniaceae) dominates cool temperate rainforest in southeastern NSW from Harrison Creek (37.5 °S), just south of the Victorian Border, to Loddon Falls northwest of Wollongong in NSW (34.5 °S) (Fig. 1). Lastly, *N. moorei,* the only other Australian member of the *Nothofagus* subgenus *Lophozonia*, dominates cool temperate rainforest at high altitudes from Barrington Tops (32 °S) to the McPherson Ranges (28 °S) in southern Queensland (Taylor *et al.*, 2005; Fig. 1). *Atherosperma moschatum* (Atherospermataceae), a tree that is usually subdominant with *N. cunninghamii* (see Fig. 3), and with *E. moorei* at Monga and *N. moorei* at Barrington Tops, occurs outside of the distribution of these species in some sites ranging from eastern Tasmania to the Tia River, north of Barrington Tops (Fig. 1).

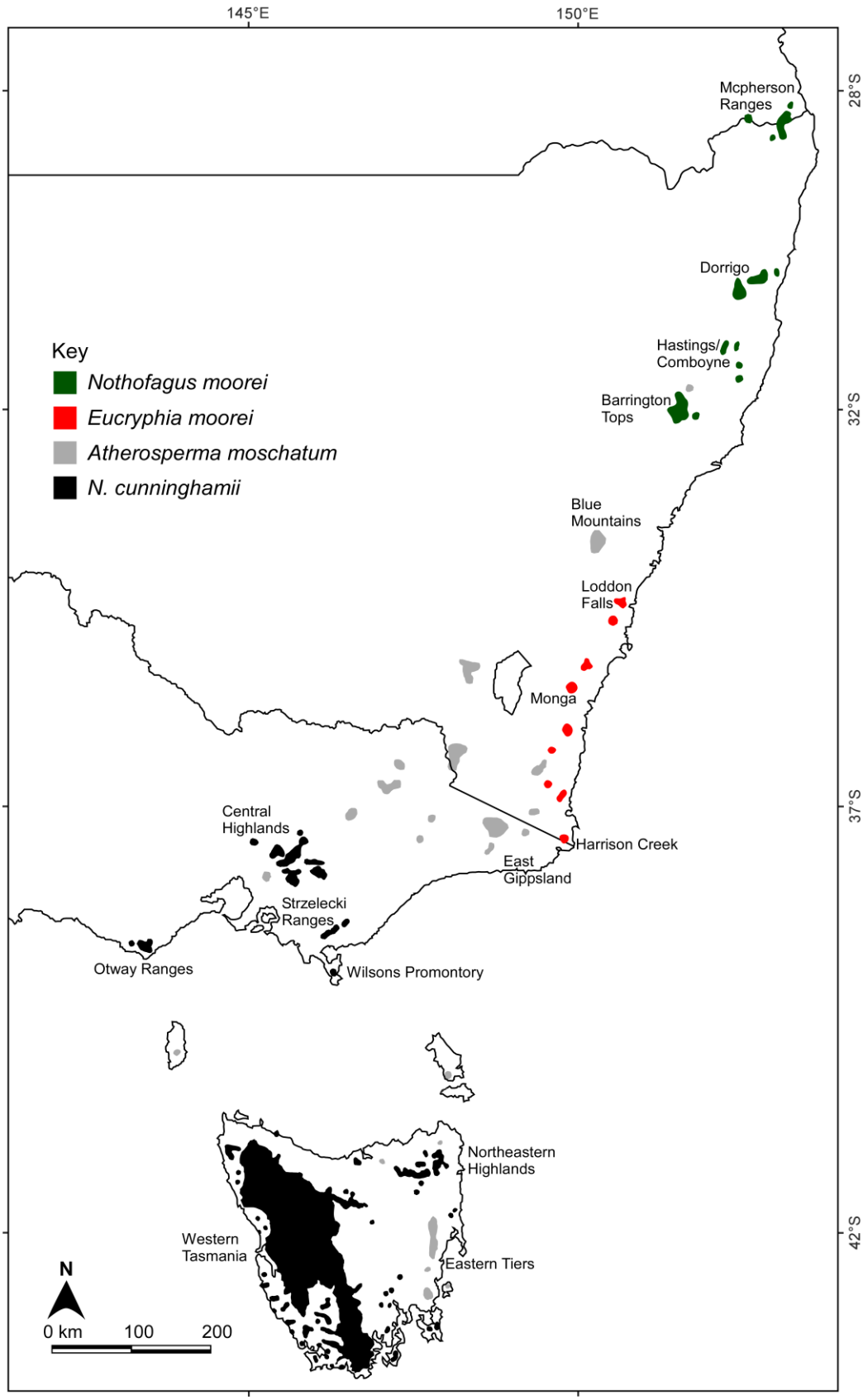

Fig. 1 Distribution of cool temperate rainforest in Australia, fractionated by the main canopy dominant species of these forests (see key). Other vegetation types other than cool temperate rainforests can occur within the distribution area of rainforests.

Fig. 2 Landscape level view of cool temperate rainforest dominated by *Nothofagus cunninghamii* (dark green) and cool temperate rainforest with a *Eucalyptus* overstorey (lighter green with dead tops) with the Denison Range in the background looking southwest from the summit of Wylds Craig, western Tasmania.

Fig. 3 Stand level view of cool temperate rainforest dominated by *Nothofagus cunninghamii* (centre) and *Atherosperma moschatum* (left, foreground) at the Rattler Ranges, northeast highlands of Tasmania. Photograph courtesy of Rob Blakers.

A number of features of the cool temperate rainforest biome make it highly suitable for investigations of the importance of mobility and adaptation/ tolerance of plants to climatic change:

1. The modern cool temperate rainforest flora is comprised of plant lineages that have had a long history in southeastern Australia. The fossil record shows that many modern species are the remnants of plant lineages that have been extensively depleted by climatically driven extinction (Jordan, 1997) from much more diverse Australian Paleogene forests and that most, if not all, modern rainforest species were in place and confined to southeastern Australia prior to the onset of the Pleistocene glaciations (Hill & Read, 1987). Therefore many modern cool temperate species must have survived the glaciations in southeastern Australia by one, or a combination of, mobility, adaptation or tolerance.

2. The fossil record indicates that across the current distribution of cool temperate rainforest the landscape has changed markedly with fluctuations in climate during the last 2.5 million years. There is evidence for at least five glaciations in southeastern Australia during the Pleistocene (McKinnon *et al.*, 2004). While ice cover was minor in southeastern Australia, during this time covering ~ 12% of Tasmania and an unknown, but certainly much smaller area of the mainland during the early Pleistocene and even less area during the last glacial maximum (Colhoun, 2002), periglacial conditions were widespread (Galloway, 1965) and glacial aridity became important by the second half of the Pleistocene (Kershaw & Nanson, 1993). The last glacial maximum saw the height of glacial climates in southeastern Australia with temperatures up to 6°C lower than present during the warmest month in Tasmania (Colhoun *et al.*, 1996) and up to 9°C in the mountains of the southeast Australian mainland (Galloway, 1965). Rainfall is believed to have declined by 50% (Colhoun, 2000) and rainfall gradients steepened, most notably between western Tasmania and the drier east of the island, so that some regions where rainforest currently occurs appear to have received precipitation as low as 500 mm per annum. This stress imposed on rainforest plants by this depression of rainfall may have been accentuated by lower CO2 concentrations (Barnola *et al.*, 1987). Fossil evidence indicates that the vegetation changed markedly throughout glacial-interglacial cycles. The climatic treeline is thought to have been depressed to near current sea-level, with more or less treeless glacial steppe or grassland extending from southern Tasmania to Barrington Tops (Hope, 1994; Sweller & Martin, 2001), though interspersed with dry forest plants such as Casuarinaceae in some areas (Williams *et al.*, 2006). At high altitudes alpine vegetation had expanded for example, in Victoria (Ladd, 1979b; McKenzie, 1997) and western Tasmania (Colhoun & Van De Geer, 1986; Gibson *et al.*, 1987). Other than parts of western Tasmania, where although temperature depression was as significant as in other regions rainfall remained within the lower limits for cool temperate rainforest (Colhoun, 2000), these changes would have made almost all of southeastern Australia inhospitable for rainforest species.

3. Cool temperate rainforest is highly tractable to detailed investigation of long-term responses to change because its low species richness but high phylogenetic disparity among species allows powerful conclusions to be made from a relatively small number of species. In addition, cool temperate rainforest, and in particular the major tree *Nothofagus cunninghamii*, has been an important model system for

understanding the past climates in southeastern Australia using fossils (e.g. Macphail, 1979; Markgraf *et al.*, 1986; McKenzie & Busby, 1992; McKenzie & Kershaw, 1997) and bioclimatic modelling (Kirkpatrick & Fowler, 1998).

4. In addition the tree and shrub species of cool temperate rainforest have a diverse range of dispersal traits.

This thesis

This thesis aims to contribute to our knowledge of the response of species to climatic change by investigating the range-wide chloroplast phylogeographies of three widespread cool temperate rainforest woody plants, *Nothofagus cunninghamii* (Nothofagaceae), *Tasmannia lanceolata* (Winteraceae) and *Atherosperma moschatum* (Atherospermataceae). These species were chosen for a number of reasons:

1. All three species are major constituents of this forest type. *Nothofagus cunninghamii* is often the dominant canopy tree of cool temperate rainforests and its distribution has been the basis of the reconstructions of rainforest distributions during the Last Glacial, and this is particularly the case in the northeast highlands of Tasmania and in Victoria. Therefore, the history of southern cool temperate rainforest has often been equated to the history of '*N. cunninghamii*'. Along similar lines, *A. moschatum* is a very common component of cool temperate rainforest and is usually subdominant tree to *N. cunninghamii* where they co-occur and can be the sole tree of cool temperate rainforest where it occurs outside the distribution of *N. cunninghamii* (Fig. 1). *Tasmannia lanceolata* is a common understorey shrub or small tree of cool temperate rainforest.

2. All three species are widespread and all co-occur across large parts of their ranges. Along with two other species (*Notelaea ligustrina* and *Pittosporum bicolor*) the three species are the most geographically extensive species that are common components of cool temperate rainforest in southeastern Australia (Table 1). These widespread distributions provide an opportunity to examine the role of mobility, adaptation and / or tolerance to understand their wide distributions.

(3) The species chosen have different dispersal mechanisms. The narrowly winged seeds of *Nothofagus cunninghamii* are apparently ineffective and are dispersed by gravity and secondarily by water (Howard, 1973; Hickey *et al.*, 1982; Tabor *et al.*, 2007). *Tasmannia lanceolata* has black fleshy fruit that are dispersed by birds (Read,

1982; Read & Hill, 1983; Cash, 1998; Borzak, 2003) and *Atherosperma moschatum* has plumose achenes that are dispersed by wind, presumably over long distances (Hickey *et al.*, 1982; Neyland & Brown, 1993). These differing dispersal traits provide an opportunity to examine whether these dispersal traits resulted in contrasting response of these species to climatic changes.

In addition, a preliminary study was also undertaken to investigate the levels and distribution of chloroplast variation within three Tasmanian endemic cool temperate rainforest plants, the tree *Phyllocladus aspleniifolius* (Podocarpaceae) and the shrubs *Olearia persoonioides* (Asteraceae) and *Telopea truncata* (Proteaceae). All three species are widespread in western Tasmania and have a disjunct occurrence in the northeast highlands of the island.

The motivation for the initiation of this study was that a molecular approach was needed to better understand the response of these cool temperate rainforests to past climatic change. Apart from western Tasmania, the fossil record is relatively uninformative as to the response in space and time of any Australian cool temperate rainforest species to climatic changes that occurred through the Pleistocene. In particular, the location of glacial refugia for cool temperate rainforest species is poorly understood. Fossil pollen data indicates that many cool temperate rainforest species have occurred through multiple glacial cycles in western Tasmania. However, the dearth of pollen records extending back beyond 11,000 years, due to gravels or soil underlying lake or swamp sediments beyond this time in southeastern Australia (Hope, 1994), means that the locations of glacial refugia for rainforest (and other forest types) in general are poorly known outside western Tasmania. The present fossil record of *N. cunninghamii*, *T. lanceolata* and *A. moschatum* from 150 kya to present are presented in Fig's 4, 5 and 6 respectively. The widespread distribution of these three species may be a result of one or a combination of all the following processes, the persistence of populations that have been isolated by fragmentation of formerly extensive forests, migration at times of suitable climate or recent long distance dispersal. The importance of differing dispersal traits in this response has not been previously investigated.

Table 1 The distribution of dicotyledon and gymnosperm trees and shrubs of the *Nothofagus cunninghamii* dominated cool temperate rainforest of southeastern Australia in the three major occurrences of this forest type (western Tasmania, northeast Tasmania and Victoria). Whether the species are common (+) or rare (r) within each region of occurrence is indicated.

	Tree or shrub species	W. Tasmania	NE Tasmania	Victoria
Dicots	*Atherosperma moschatum*	+	+	+
	Notelaea ligustrina	+	+	+
	Nothofagus cunninghamii	+	+	+
	Pittosporum bicolor	+	+	+
	Tasmannia lanceolata	+	+	+
	Telopea truncata	+	+	
	Aristotelia peduncularis	+	+	
	Pimelea drupacea	+	+	
	Tetracarpaea tasmanica	+	r	
	Trochocarpa (3 spp.)	+	r	
	Olearia (2 spp.)	+	r	
	Acradenia frankliniae	r		
	Agastachys odorata	+		
	Anodopetalum biglandulosum	+		
	Anopterus glandulosus	+		
	Archeria (2 spp.)	+		
	Cenarrhenes nitida	+		
	Dracophyllum milliganii	r		
	Epacris mucronulata	+		
	Eucryphia (2 spp.)	+		
	Leptospermum riparium	+		
	Lomatia (2 spp.)	+/r		
	Nothofagus gunnii	+		
	Orites (2 spp.)	+		
	Prionotes cerinthoides	+		
	Pseudopanax gunnii	r		
	Richea (3 spp.)	+		
	Persoonia arborea			r
	Wittsteinia vacciniacea			r
Gymnosperms	*Podocarpus lawrencii*	+	r	r
	Phyllocladus aspleniifolius	+	+	
	Athrotaxis (2 spp.)	+		
	Diselma archeri	+		
	Lagarostrobos franklinii	+		
	Phaerosphaera hookeriana	r		

Structure of this thesis

The three range-wide chloroplast phylogeographic studies are presented as self-contained chapters. Each of these chapters is presented in the style of scientific journal articles, and contains an introduction to the relevant literature and outlines the potential contribution that the experimental work can make in an international or Australian context. A discussion detailing the findings of the work and its contribution to the research field is also presented. Chapter 2 describes the chloroplast phylogeography of *N. cunninghamii* and focuses on whether this species was able to survive through glacials in multiple regions, including eastern Tasmania and parts of southern Victoria that would have been inhospitable for the species based on our understanding of its ecological tolerance range based on its current distribution. This chapter has been published in a refereed journal (Worth *et al.*, 2009) and is presented exactly as published except for minor changes to standardise nomenclature throughout the thesis, and the removal of the abstract and the collation of the references in the general bibliography of the thesis. Chapter 3 describes the chloroplast phylogeography of *T. lanceolata* and focuses on the consequences of its bird-dispersed fruit on the genetic structuring of its chloroplast variation across its range and the implications for our understanding of the history of dispersal of this species. Chapter 4 describes the chloroplast phylogeography of *A. moschatum* and focuses on understanding the importance of the species wind-dispersal in the widespread distribution of this species and the chloroplast diversity observed. Chapter 3 and 4 are experimental chapters that are in preparation for submission. The last research chapter, Chapter 5, is not in style of a scientific journal article but rather presents the methods and results of a preliminary investigation of chloroplast diversity in three Tasmanian endemic plants. Chapter 6 discusses the major findings of this thesis and how they relate to present knowledge and suggests some directions for future research in understanding of cool temperate rainforest.

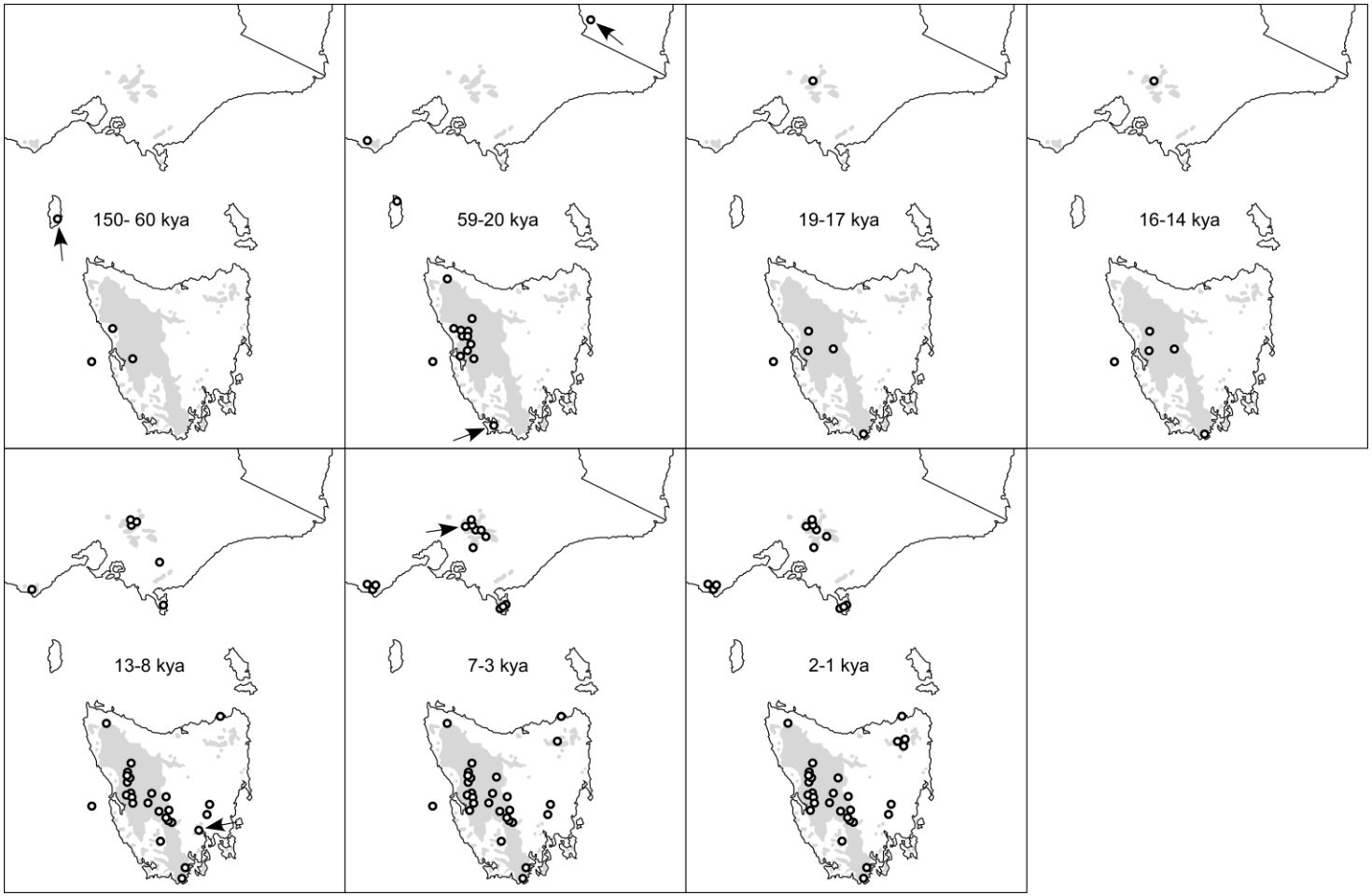

Fig. 4 The fossil record of *Nothofagus cunninghamii* (Nothofagaceae) with the modern distribution of *N. cunninghamii* shown in grey. The arrows indicate records that contained macrofossils of the species. Map compiled from (Caine & Jennings, 1968; Howard & Hope, 1970; Hope, 1974; Macphail & Jackson, 1978; Ladd, 1979a; Macphail, 1979; Colhoun, 1980; Colhoun *et al.*, 1982; Colhoun & Moon 1984; Macphail, 1984; Colhoun, 1985; Macphail & Colhoun, 1985; Colhoun & Van De Geer, 1986,1987; Colhoun *et al.*, 1989; Van De Geer *et al.*, 1989; Colhoun *et al.*, 1991a; Colhoun *et al.*, 1991b; Jordan *et al.*, 1991; Jordan *et al.* unpublished; Lloyd & Kershaw, 1997; Macphail, 1991; Colhoun, 1992; Colhoun *et al.*, 1992; Colhoun *et al.*, 1993; D'Costa *et al.*, 1993; Harle *et al.*, 1993; Van Der Geer *et al.*, 1994; Colhoun, 1996; Thomas, 1996; Thomas & Kirkpatrick, 1996; McKenzie, 1997; Colhoun & Van Der Geer, 1998; Dodson *et al.*, 1998; Colhoun *et al.*, 1999; Harle *et al.*, 1999; Hopf *et al.*, 2000; McKenzie, 2002; McKenzie & Kershaw, 2000; Anker *et al.*, 2001; Dodson, 2001; Rowell *et al.*, 2001; McKenzie & Kershaw, 1997; McKenzie & Kershaw, 2004; Fletcher & Thomas, 2007).

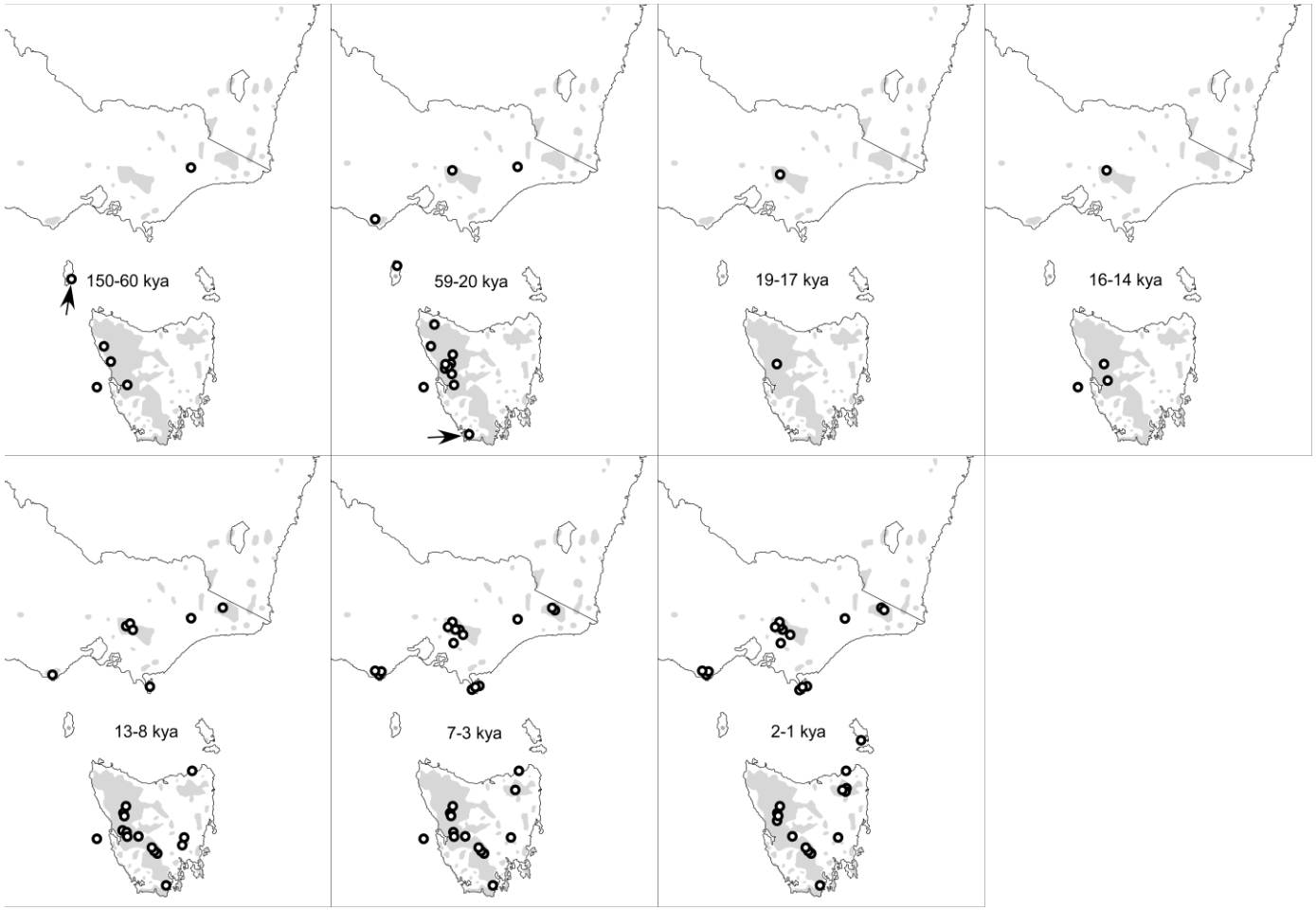

Fig. 5 Fossil record of *Tasmannia lanceolata* (Winteraceae) with the current distribution of the species shown in grey. Two macrofossil sites of the species are indicated by arrows. The pollen records of *Tasmannia* on mainland Australia may be other species of the genus not present in Tasmania. Map compiled from (Howard & Hope, 1970; Hope, 1974; Macphail, 1975; Macphail & Jackson, 1978; Ladd, 1979a; Ladd, 1979c; Ladd, 1979b; Colhoun, 1980; Colhoun *et al.*, 1982; Colhoun, 1985; Colhoun & Van De Geer, 1986,1987; Colhoun *et al.*, 1989; Van De Geer *et al.*, 1989; Colhoun *et al.*, 1991a; Jordan *et al.*, 1991; Jordan *et al.* unpublished; Colhoun *et al.*, 1993; D'Costa *et al.*, 1993; Harle *et al.*, 1993; Van Der Geer *et al.*, 1994; Thomas, 1996; Thomas & Kirkpatrick, 1996; McKenzie, 1997; McKenzie & Kershaw, 1997; Colhoun & Van Der Geer, 1998; Dodson *et al.*, 1998; Colhoun *et al.*, 1999; McKenzie & Kershaw, 2000; Anker *et al.*, 2001; McKenzie, 2002; Ladd & Clarke, 2004; McKenzie & Kershaw, 2004; Kershaw *et al.*, 2007).

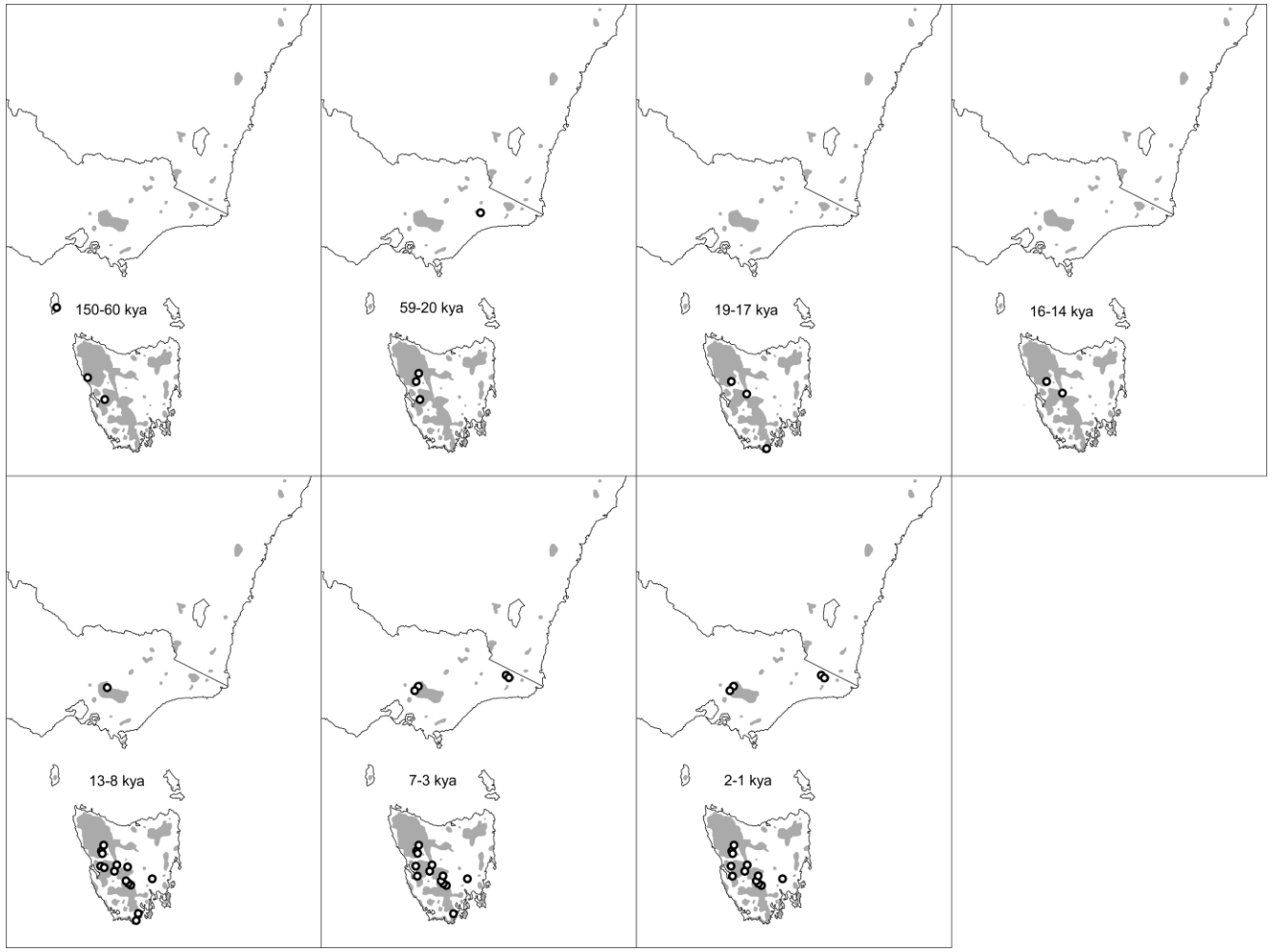

Fig. 6 Fossil record of *Atherosperma moschatum* with the current distribution of the species shown in grey. Only one macrofossils of this species has been found in ~ 1 million year old sediments at Regatta Point, western Tasmania (not shown). Map compiled from (Jordan et al. unpublished; Ladd, 1979c; Ladd, 1979b; Macphail, 1979; Colhoun, 1980; Macphail & Colhoun, 1985; Colhoun & Van De Geer, 1986; Van De Geer *et al.*, 1989; Colhoun *et al.*, 1991a; Harle *et al.*, 1993; McKenzie, 1997; Colhoun & Van Der Geer, 1998; Colhoun *et al.*, 1999; Harle *et al.*, 1999; Hopf *et al.*, 2000; Anker *et al.*, 2001; McKenzie, 2002; Kershaw *et al.*, 2007).

Chapter 2: The major Australian cool temperate rainforest tree *Nothofagus cunninghamii* withstood Pleistocene glacial aridity within multiple regions: evidence from the chloroplast

Introduction

There is continuing debate on how plants and animals have survived through past major climatic changes, enabling the assembly of temperate communities during the current interglacial. Many authors, particularly using fossil pollen data from the northern hemisphere, have proposed that temperate forest species migrated very rapidly from a few distinct refugia located in regions that escaped the major climatic changes of the glaciations (Huntley & Birks, 1983; Hewitt, 1996). In this model, current temperate tree populations outside known glacial refugia are thought to have been established during the postglacial period via migration over thousands of kilometres (Jackson & Overpeck, 2000), and across both land and sea barriers (Davis *et al.*, 1986; Webb, 1987; Bennett, 1995). More recently, both macrofossil and phylogeographic evidence have been used to argue for more complex histories involving expansion from multiple refugia (e.g. Stewart & Lister, 2001; McLachlan *et al.*, 2005; Petit *et al.*, 2008). This has resulted in the identification of refugia in locations unexpectedly close to regions where glacial climates had major effects on the environment. The locations of these refugia often conflict with present: knowledge of the tolerance range and/or adaptive abilities of species; models of glacial climatic conditions (e.g. temperature, aridity, permafrost and ice cover); interpretations of pollen evidence; and predictions of biogeographical histories of species from current distribution patterns. Such refugia are sometimes referred to as "cryptic" (e.g. Stewart & Lister, 2001; Provan & Bennett, 2008). The southern hemisphere provides opportunities to better understand how important large-scale postglacial migrations and/or expansions from multiple glacial refugia may have been in shaping the current forests of the temperate zone.

Temperate rainforests of the southern hemisphere occur from latitudes 28°S to 55°S in South America, New Zealand and Australia. In each of these regions,

palaeoecologists have proposed that these forests recovered from glacial climates by expansion from multiple refugia without extensive range shifts (Macphail & Colhoun, 1985; McGlone, 1985; Markgraf *et al.*, 1995). In southeastern Australia, most areas of cool temperate rainforest, as defined by Webb (1959), are dominated by *Nothofagus cunninghamii* (Nothofagaceae). These rainforests have a widespread but discontinuous distribution in the wettest and most fire protected regions (Hill *et al.*, 1988) and are surrounded by more extensive sclerophyll forests.

Many authors have argued that arid conditions during Pleistocene glaciations would have made almost all of southeastern Australia inhospitable for cool temperate rainforest (e.g. Hope, 1994; Hill, 2004). The available pollen evidence in southeastern Australia from the Last Glacial Maximum (LGM) 18,000 years ago indicates a more or less treeless landscape dominated by glacial steppe vegetation probably to present sea-level. Pollen evidence identifies rainforest tree LGM survival in only two places: the western half of Tasmania, where the coastal plains exposed by depressed LGM sea-levels may have provided suitable habitat (Kiernan *et al.*, 1983; Colhoun, 2000) and the Central Highlands of Victoria (McKenzie, 1997).

There has been particular controversy about whether two regions of southeastern Australia contained glacial refugia: the highlands of northeast Tasmania and southern Victoria (Fig. 1). Depauperate cool temperate rainforest communities are reasonably extensive in these regions (Busby, 1984) but they receive less than 50% of the precipitation of the wetter parts of western and southern Tasmania (Nunez, 1978) where the most geographically extensive cool temperate rainforests exist.

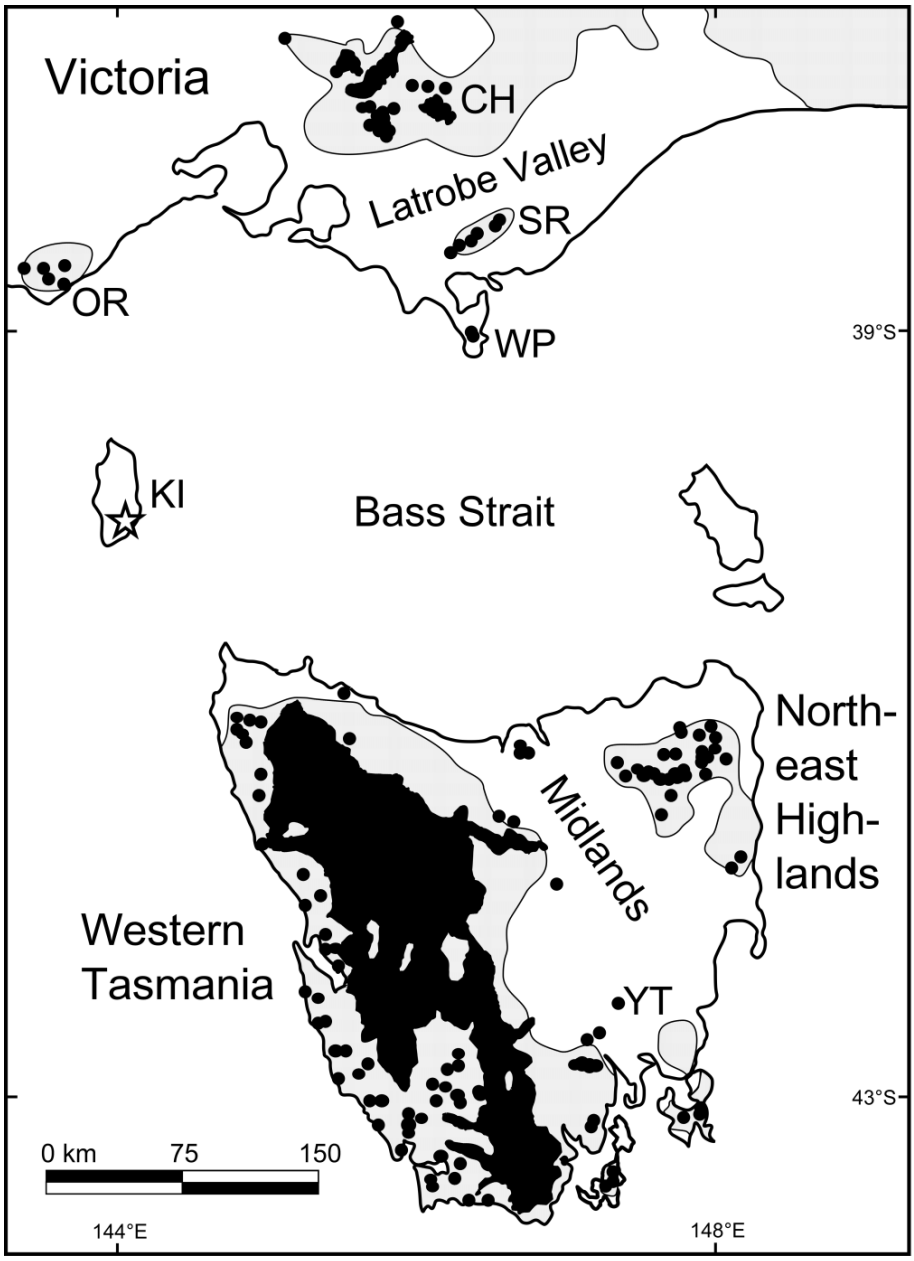

Fig. 1 Distribution of *Nothofagus cunninghamii* cool temperate rainforest (black areas) in southeastern Australia. Grey stippled areas indicate regions in Tasmania and Victoria receiving over 50 mm of precipitation during the driest month. Geographical names mentioned in text: Victorian Central Highlands (CH), Strzelecki Ranges (SR), Otway Ranges (OR), Wilsons Promontory (WP), King Island (KI), Yarlington Tier (YT). Some major dry land and sea barriers are also shown. Star on KI indicates location of macrofossil site of *N. cunninghamii* dated at ~ 110,000 years before present (G. J. Jordan unpublished).

The available geomorphological and pollen based evidence indicate extensive glacial aridity during the LGM in northeast Tasmania and southern Victoria (Galloway, 1965; Bowler, 1982; Colhoun, 2002) with the Australian desert thought to have extended to within 100 km of current cool temperate rainforest populations (Bowden, 1983; Hill & Bowler, 1995). In northeast Tasmania palaeoclimatic modelling can reconstruct conditions favourable for LGM survival of cool temperate rainforest only by invoking eastern Tasmanian climates with similar rainfall to the present (Kirkpatrick & Fowler, 1998), contrary to the evidence for much higher aridity. Even this modelling could only identify refugia in the wettest part of eastern Tasmania, Blue Tier. An alternative explanation involving dispersal rather than glacial refugia for the occurrence of rainforest in northeast Tasmania must invoke Holocene dispersal across > 150 km from the nearest documented refugia in western Tasmania, a scenario that has been considered unlikely because of the low dispersal capacity of many rainforest species (Dodson & Ono, 1997; Kirkpatrick & Fowler, 1998), particularly *N*. *cunninghamii*. Herein lies the conundrum: the areas where cool temperate rainforest currently occur can only have arisen from *in situ* glacial refugia if our understanding of glacial climates is wrong, and/or the ecological tolerance range of species during past climatic changes was greater than would be predicted from their modern distribution. However, if rainforest did not survive in multiple regions extensive range shifts must be invoked (Jordan, 2003). Therefore, the cool temperate rainforest system of southeastern Australia provides an opportunity to test the relative roles of multiple refugial survival and postglacial dispersal.

This study aims to address this conundrum by investigating the chloroplast DNA phylogeography of the dominant cool temperate rainforest tree in southeastern Australia, *Nothofagus cunninghamii* (Hook.) Oerst. The current distribution of chloroplast DNA (cpDNA) haplotype variation across a species' range can provide independent evidence for the history of genetic exchange by seed and isolation of populations (Schaal *et al.*, 1998). Isolated populations may differentiate over time and, through genetic drift, form distinct genetic lineages. Dispersal can result in the territorial expansion of genetic lineages (Avise, 1994) and the sharing of lineages between populations. Chloroplast DNA phylogeographic studies have been used to investigate the location of glacial refugia and migration histories of temperate forest mostly in the northern hemisphere (e.g. Soltis *et al.*, 1997; Okaura & Harada, 2002; Petit *et al.*, 2003). Although range-wide cpDNA phylogeographic studies have been

completed in some southeastern Australian sclerophyll forest *Eucalyptus* species (Byrne & Moran, 1994; Freeman *et al.*, 2001), this study is the first cpDNA phylogeography of a widespread cool temperate rainforest species in Australia. This study assesses the contributions of multiple glacial refugia to postglacial recovery of *N. cunninghamii*. Specifically we address whether glacial survival occurred outside putative refugia in coastal western Tasmania and the central highlands of Victoria.

Materials and Methods

The study species

Nothofagus cunninghamii (myrtle beech) is a long lived, evergreen, monoecious, wind pollinated tree reaching 50 m in height (Curtis, 1967), but at the altitudinal maximum of its range may be reduced to a compact shrub under 50 cm. The species is remarkably uniform in morphology across its range apart from variation in leaf size, which is strongly correlated with summer temperatures (Jordan & Hill, 1994). Seed is gravity-dispersed, generally one tree height from the mother tree (Howard, 1973; Hickey *et al.*, 1982; Tabor *et al.*, 2007), but may be dispersed downstream in water courses (Howard, 1973). The species' distribution is broken by some major dry land and sea barriers (Howard & Ashton, 1973; Fig. 1). Across its range this species is confined to cool, humid climates where rainfall exceeds ~ 1000 mm per annum with at least 50 mm rainfall during the driest month (Jackson, 1965; Busby, 1986; Lindenmayer *et al.*, 2000). The small populations outside this climatic range (e.g. Yarlington Tier; Fig. 1) are all special topographic sites with precipitation supplemented by ground water and/or cloud stripping (Harle *et al.*, 1993). *Nothofagus cunninghamii* does not currently co-occur with any other species of its subgenus, *Lophozonia*. Its sister species, *N*. *moorei*, is restricted to mountain ranges of northern New South Wales and southern Queensland (Busby, 1986), ~ 780 km north of the northernmost population of *N. cunninghamii*. The western Tasmanian endemic, *N*. *gunnii* (subgenus *Fuscospora*), sometimes co-occurs with *N*. *cunninghamii* but the two species do not hybridise (Hill & Read, 1991).

Sampling

Fresh leaves were collected from 342 adult trees (327 stands) of *N. cunninghamii* in natural populations including nearly all known parts of the species' distribution apart from some remote parts of western Tasmania. Only one tree was sampled from most

stands, but up to three widely spaced individuals were sampled at some locations, including some very isolated populations (see Appendix 1 and 2). Latitude, longitude and altitude information were recorded for each sample collected. Tree form was noted. Five individuals of *N. moorei* were also sampled from the northern (Lamington National Park, Springbrook National Park and Bar Mountain) and southern extremes of this species' range (two samples from Barrington Tops National Park ~ 490 km south of the northern populations of this species). For use as outgroups, leaf samples of the New Zealand endemic *N. menziesii*, and the Chilean species *N. glauca,* were obtained from the Royal Tasmanian Botanical Gardens, Hobart, Tasmania. Phylogenies based on morphology, nuclear DNA and chloroplast DNA each indicate that *N. moorei* is the sister of *N. cunninghamii*, that *N. menziesii* is sister of this clade and that the resulting clade is sister to a small clade containing *N. glauca* (Manos, 1997). These samples were analysed in two groups: 1) a range-wide survey whereby a haplotype phylogeny was created from chloroplast sequence and PCR-RFLP data using 213 *N. cunninghamii* samples from across the distribution of the species and all outgroups; and 2) a fine-scale study of haplotype distribution that used three PCR-RFLP characters and screened 149 samples from the northeast highlands of Tasmania (including 20 samples used in the previous study). For this study individual trees were sampled a minimum of ~ 1 km apart.

Molecular methods

Total genomic DNA was extracted from 1 g of adult leaves, following the CTAB protocol of Doyle & Doyle (1990). DNA concentration and purity was assessed using agarose gel electrophoresis with ethidium bromide staining and comparison with a standard molecular weight marker (Lambda *Hind*III). DNA concentration was standardized at 5 ng per μL.

Sixteen regions of cpDNA were amplified using universal primers (*trnD–trnT*, *trnS–trnf*$_M$, *trnK–trnQ*, *rpoC1–trnC*, *trnV–rbcL*, *rpl23–psbA3*, *atpH–atpI*, *atpI–rpoC2*, *rpoC2*-f–*rpoC2*-r, *orf184–petA*, *petA*-f–*psbE*-r, *clpp–psbB*, *psbB–petB*, *petB–petD*, *trnH–trnK* and *trnK–trnK*) (Demesure *et al.*, 1995; Dumolin-Lapegue *et al.*, 1997b; Grivet *et al.*, 2001). All PCR reactions were performed in a total volume of 25 μl containing 2.5 mM $MgCl_2$; 100 μg/mL of Bovine Serum Albumin; 80 μM each of dATP, dCTP, dGTP and dTTP; 5 pM of each primer; 1 x PCR buffer (67 mM Tris-HCl, 16.6 mM $(NH_4)_2 SO_4$, 0.5% Triton X-100 and 5 μg of gelatin); two units of *Taq* DNA polymerase; and approximately 10

ng of genomic DNA. PCR amplification was performed by a MJ Research PTC-225 Tetrad thermocycler (GMI, Inc., Minn., USA) as follows: an initial melt of 4 min at 94°C; 30 cycles of 45 sec at 92°C, 45 sec at annealing temperature (see Appendix 3), 4 min at 72°C; and a final extension for 10 min at 72°C. PCR products were digested with a range of restriction enzymes in a total reaction volume of 20 µl containing 5-10 µL of PCR product. The products of the restriction digests were size fractionated in a 2.2% agarose gel in TBE at 100 V for 90 min. Restriction fragment length polymorphisms (RFLPs) were identified visually by comparing restriction fragment patterns between samples.

Screening of eight *N. cunninghamii* samples (representing all major parts of the species range) and 150 fragment/restriction endonuclease combinations (using *Taq*I, *Hinf*I, *Alu*I, *Dpn*II, *Hae*III, *Hinp1*I, *Rsa*I, *Dde*I, *Msp*I, *Hph*I, *Nco*I, *Ssp*I, *Ase*I, *Sty*I, *Nci*I, *Dra*I, *Cla*I, *BstU*I, *EcoR*V) revealed one RFLP. Screening of 48 or 72 samples and 16 fragment/restriction endonuclease combinations detected two additional RFLPs. All samples for the range-wide survey were screened for the three fragment/restriction endonuclease combinations that detected polymorphisms.

Owing to the paucity of cpDNA variation detected using PCR/ RFLP, cpDNA fragments of all samples for the range-wide survey were sequenced. These were partial sequences of the intergenic spacer regions *petN1–psbM2*R, *psbM2–trnD* (Lee & Wen, 2004), *trnS–trnf*$_M$ (Demesure *et al.*, 1995), and *trnL–trnF* (Taberlet *et al.*, 1991) and the intron between *rps16*/1 F–*rps16*1 R (Nishizawa & Watano, 2000). PCR conditions were as follows: *petN1–psbM2*R and *psbM2–trnD*, denaturation for 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C; *trnS–trnf*$_M$, denaturation for 4 min at 94°C, followed by 30 cycles of 45 sec at 92°C, 45 sec at 62°C, and 4 min at 72°C; *trnL-trnF*, denaturation for 1 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 50°C and 45 sec at 72°C; *rsp*16/1 F–*rsp*161 R, denaturation for 3 min at 95°C, followed by 25 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. All had a final extension for 10 min at 72°C except *trnL–trnF* with a final extension of 7 min at 72°C. Before sequencing, PCR products were purified using the Qia-Quick PCR purification kit (QIAGEN Pty Ltd, Vic, Australia). Sequencing reactions were performed using a Beckman Coulter Quick Start Kit following a modified protocol using 0.64 µl of 5 µM primer, and 6 µl of purified PCR product in a final volume of 10 µl. Sequence reactions were analysed using a Beckman Coulter CEQ 2000 automated

sequencer (Beckman Coulter, Inc. CA, USA). Polymorphisms detected in only one sequencing reaction were checked by repeating the PCR and the sequencing reaction. In all cases where unexpected haplotype distributions were found (e.g. *N. moorei*), sequences and PCR-RFLP analyses were repeated, and samples rechecked.

For the fine-scale study of northeast Tasmanian haplotypes, restriction endonucleases that would enable the quick and easy identification of individuals carrying either the C1 or NE1 haplotypes (see below) were identified using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/index.php). The restriction endonuclease *Hae*III cut the *trnL–trnF* fragment of C1 individuals once, and zero times in NE1 individuals as a result of a 17 base pair (bp) deletion. The endonuclease *Hpy*188III was found to distinguish the *psbM2–trnD* fragment of individuals carrying NE1 haplotype due to an extra restriction site in this haplotype. For this analysis a new internal reverse primer was developed (5'…CCGGGACTCGTCTTTATCATACTTC…3') that amplified a cpDNA fragment approximately 540 bp in length compared to the original approximately 1200 bp fragment. This allowed better separation of the polymorphic fragments that differed by 77 bp in length between C1 and NE1 haplotypes. All 149 samples were screened with these two new endonuclease/ fragment combinations and the previously identified *atpI–rpoC2* fragment/*Taq*I combination.

Phylogenetic relationships of haplotypes

Evolutionary relationships between haplotypes (including *N. glauca*, *N. menziesii* and *N. moorei*) were investigated by maximum parsimony analysis undertaken using the program PAUP* version 4.0b10 (Swofford, 2000). PCR-RFLP polymorphisms, single nucleotide polymorphisms and insertions/deletions were scored as binary characters, except for two indel variants (characters 1 and 2; Table 1), which were scored as a multi-state character with three states. In addition, parallel variation was seen in two adjacent base pairs (a doublet). This was treated as a single character (character 20, Table 1).

Parsimony analysis was undertaken using a heuristic search with 1000 replicates of stepwise, random branch swapping addition sequence followed by tree-bisection-reconnection (TBR). Due to significant levels of homoplasy of some characters (in particular characters 19 and 20; Table 1), all characters were reweighted iteratively by the maximum value of their rescaled consistency index (Farris, 1969). A strict

consensus of all the shortest trees found using this procedure was constructed. Branch support was assessed by bootstrap analysis (Felsenstein, 1985) with 1000 heuristic search pseudo-replicates using the same search parameters as those in the parsimony analysis. *Nothofagus glauca* and *N. menziesii* were used as outgroups in all searches based on the topologies identified by (Manos, 1997).

Spatial clustering and regional haplotype diversity

Within the 213 samples collected across the range of *N*. *cunninghamii* the spatial structuring of haplotypes was investigated. The single nearest geographic neighbour for each sample was determined using a specially written macro in SAS 9.1 (SAS Institute Inc., Cary, NC, USA). This program also performed a permutation test (Manly, 1997) with 10,000 randomised repeats testing whether the nearest neighbour of each sample was more often of the same haplotype than expected by chance (a proxy of spatial structure). This was carried out across all samples and within each region (Victoria, eastern Tasmania and western Tasmania). This procedure was also applied to the 149 sample set from northeast Tasmania in the fine-scale study.

Haplotype diversity was also compared across these regions through rarefaction analysis (Simberloff, 1979). The three regions were randomly sub-sampled to the size of the least sampled region ($N = 26$) 10,000 times. Differences in haplotype richness were then tested using a permutation test (Manly, 1997) with 10,000 randomised repeats, programmed in SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Similar analyses were performed comparing low, medium and high altitude samples in western Tasmania, rarefying each to 43 samples. Low altitude was defined as below 150 m above sea-level (masl), the estimated tree line during the Last Glacial Maximum (Colhoun, 1985a; Gibson *et al.*, 1987). High altitude was defined as above 500 masl, which divided the remaining samples approximately evenly and is far above any plausible estimate of LGM tree lines given that all palaeoclimatic estimates show LGM temperatures at least 5°C lower than present (Galloway, 1965; Colhoun, 1985a, 2000).

Table 1 *Nothofagus cunninghamii* and *N. moorei* haplotypes with cpDNA sequence and PCR/RFLP characters shown in comparison to the *N. cunninghamii* C1 haplotype. The characters unique to *N. glauca* and *N. menziesii* are not shown. Indels are shown as insertions (I) or deletions (D) as determined from the C1 haplotype. Order of haplotypes follows their order in the strict consensus MP tree.

Haplotype	*rps16* intron				*petN1-psbM2*R spacer								*psbM2-trnD* spacer							*trnS-trnfM* spacer						*trnL-trnF* spacer									PCR-RFLP			
Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
C1	.	.	C	C	.	.	.	.	T	T	G	A	T	A	A	C	C	T	T	TC	.	T	A	A	.	T	.	.	A	G	G	A	.	G	0	0	0	0
WT1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.
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WT17	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.
WT18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	GA	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.
V1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.
V2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	GA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.
V3	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	GA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.
WT4	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
WT 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	GA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
WT6	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
WT7	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
WT9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.
WT10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
WT8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
WT11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	GA	.	.	.	.	.	.	.	.	T	T	C	.	.	.	.	.	.	.
WT12	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	T	T	C	.	.	.	.	.	.	.
WT13	.	I	T	.	.	.	I	D	.	.	.	.	.	.	C	.	.	.	.	GA	.	.	.	.	.	C	.	.	.	T	.	.	A	.	.	.	.	.
WT14	.	.	.	.	.	.	.	D	.	.	.	C	.	.	.	A	.	.	G	.	.	.	.	.	I	C	.	.	.	T	.	.	.	.	.	.	.	.
N. moorei	.	.	.	.	.	.	.	D	.	G	.	C	.	.	.	A	A	.	G	.	.	.	.	.	.	C	.	.	G	T	.	.	.	.	.	.	.	1
WT15	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	A	A	.	G	.	.	G	.	.	.	C	.	.	.	T	C	.	.	.	.	.	.	.
WT16	.	.	.	.	.	.	.	D	C	.	.	.	.	.	.	A	A	.	G	.	.	.	.	C	.	C	I	.	.	T	.	.	.	.	.	.	.	.
NE1	.	.	.	.	.	.	.	D	.	.	.	.	G	.	.	A	A	.	.	GA	.	.	.	.	.	C	.	.	.	T	.	D	.	.	.	1	.	.
N. glauca	.	.	.	.	D	.	.	D	.	.	.	.	.	.	.	A	A	.	G	GA	.	.	.	C	.	C	.	.	.	T	.	.	.	1	.	.	.	.
N. menziesii	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	A	A	.	.	GA	.	.	.	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.

Results

cpDNA variation and phylogenetic relationships

A total of 35 polymorphisms defining 23 different haplotypes (Table 1) were found within the 213 samples of *N*. *cunninghamii*. Three PCR-RFLP polymorphisms were identified after restriction digest of three fragments (*trnK*–*trnK*, *atp*I–*rpoC*2 and *orf*184–*petA*) all using the restriction endonuclease *Taq*I (Table 2).

Twenty single nucleotide polymorphisms (six transitions and 14 transversions), one doublet sequence polymorphism and eleven indels from one to 23 bp in length were identified from a total aligned sequence length of 2164 bp per sample. A total of 39 other polymorphisms were identified when adding samples of *N. moorei*, *N. menziesii* and *N. glauca* (data not shown). Two single nucleotide polymorphisms and one RFLP polymorphism were unique to the five *N*. *moorei* samples, which were all of the same haplotype (Table 1). All unique sequences were deposited in Genbank (Table 3).

Table 2 Molecular weights in base pairs (bp) of the polymorphic fragments obtained using the variable fragment/ restriction endonuclease combinations found in *N. cunninghamii* and the five *N*. *moorei* samples.

Polymorphic Fragments	Character number	Primary state[a] →Variant (bp)	Haplotype/ species
*trn*K–*trn*K-(*Taq*I)2[b]	35	540→ 420	V1,V2,V3, *N*. *glauca*
*orf*184–*pet*A-(*Taq*I)1	36	620→ 1030	WT1, WT2, WT3,WT17,WT18
*atp*I–*rpo*C2-(*Taq*I)1	37	870→ 1010	NE1
*atp*I–*rpo*C2-(*Taq*I)3	38	250→ 330	*N*. *moorei*

[a] Major pattern determined from the C1 haplotype

[b] Polymorphic fragment number (largest to smallest) after size fractionation

Table 3 Genbank accession numbers of each unique sequence and the aligned length in base pairs obtained for each of the five fragments sequenced in *N*. *cunninghamii*. Variation in aligned sequence length shown is due to indel polymorphisms.

Fragment	Length (bp)	GenBank accession #
*rsp*16 intron	363 to 365 bp	EF101599-EF101602
*petN*1-*psbM*	399 to 411 bp	EF101589-EF101595 + EU921269
psbM-trnD	525 bp	EF101578-EF101584 + EU921267-EU921268
trnS-trnfM	420 to 443 bp	EF101606-EF101611
trnL-trnF	398 to 420 bp	EF095753-EF095761

Parsimony analysis with iterative reweighting of the full cpDNA dataset yielded 412 most parsimonious trees based on 20 informative characters (Figs 2 & 3). Haplotypes of *N. cunninghamii* and *N. moorei* formed a monophyletic group. Within *N*. *cunninghamii*, 19 haplotypes formed a large, partially resolved clade (Clade 1) with good support (bootstrap percentage, BP = 75%; Fig. 2). Within Clade 1, the most common and widespread haplotype (C1), occurred in a subclade with a BP of 63% (subclade A; Fig. 3). Subclade A also included 11 uncommon haplotypes found only in western Tasmania (WT1-WT7, WT9, WT10, WT17 and WT18), and a clade (62% BP support) of three haplotypes found only in the central highlands of Victoria (V1–V3). The remaining haplotypes within Clade 1, haplotypes WT8, WT11, WT12 and WT13, fell outside subclade A (Fig. 3). An unsupported sister clade to Clade 1 (Clade 2; Fig. 2) contained three additional western Tasmanian haplotypes. Surprisingly, the single *N. moorei* haplotype was nested within this clade (Fig. 2), with *N*. *moorei* differing from *N. cunninghamii* haplotype WT14 by five characters. A single haplotype unique to northeast Tasmania (NE1) fell outside both Clades 1 and 2.

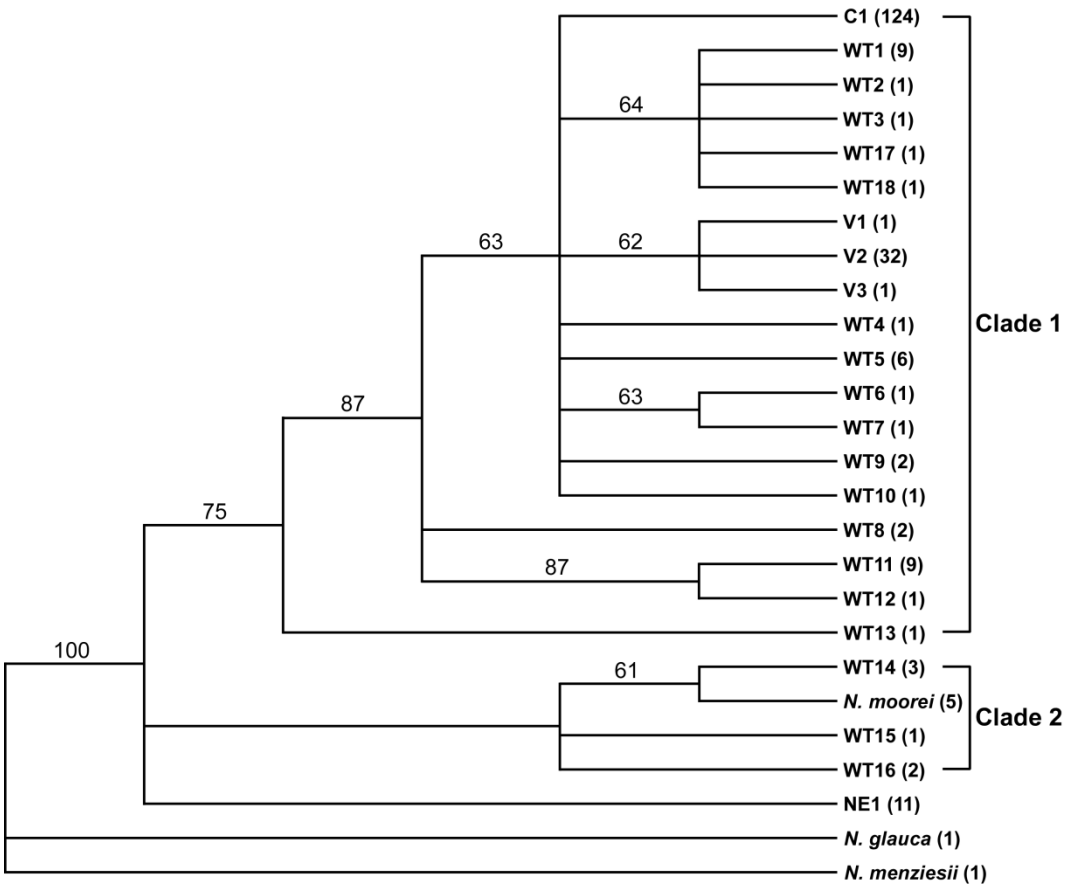


Fig. 2 Inferred phylogenetic relationships of all haplotypes found in *Nothofagus cunninghamii* and *N. moorei* based on maximum parsimony (MP) analysis, with iterative reweighting of all characters. This MP strict consensus was based on 412 most parsimonious trees (L = 70.34, CI = 0.97, RI = 0.96, RC =0.93). Bootstrap values above 60% are shown above branches. Haplotype names indicate the region in which they were found (WT, western Tasmania; V, Victoria; NE, northeast Tasmania) except for the most widespread haplotype C1. The number of occurrences for each haplotype is shown in brackets after the haplotype name.

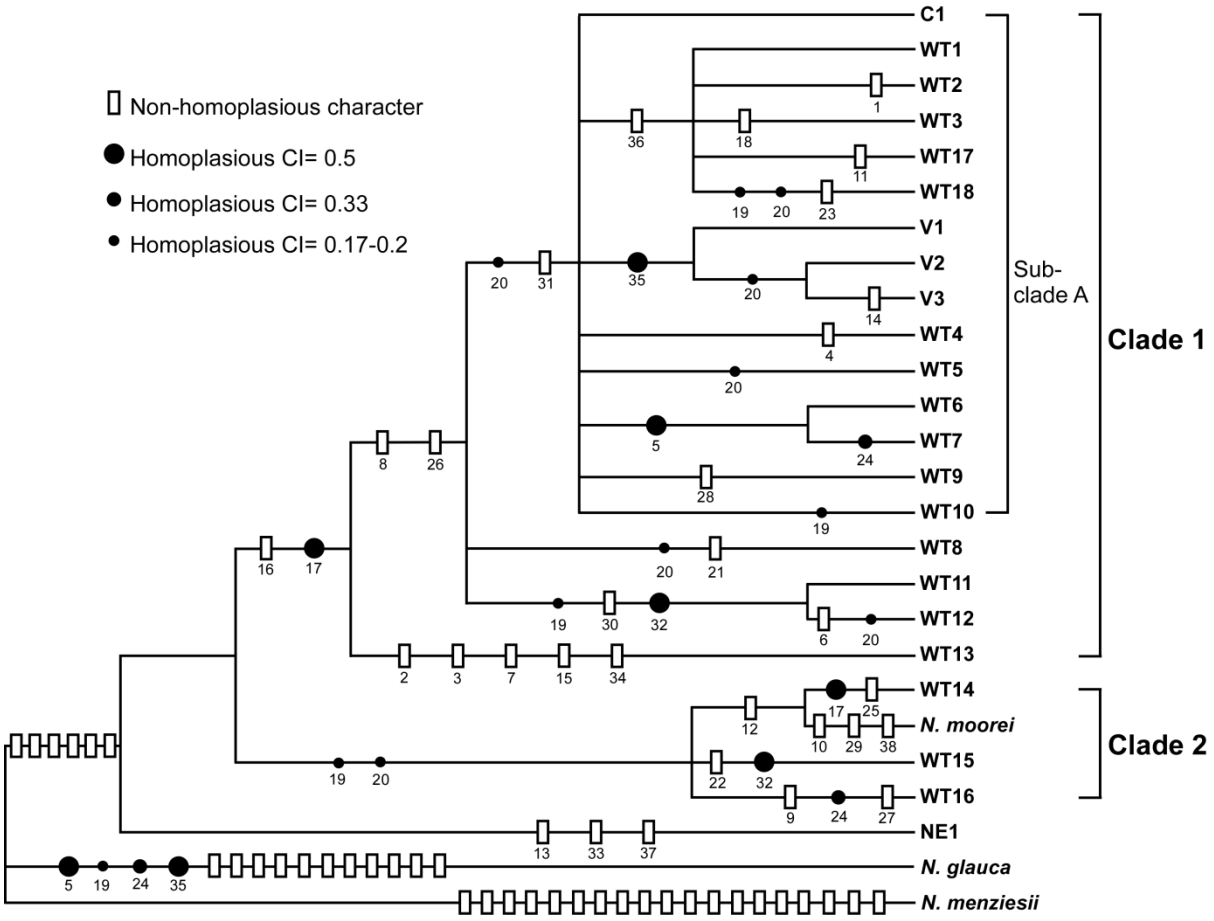


Fig. 3 One of 412 most parsimonious trees with iterative reweighting. Numbers below branches indicate the *N*. *cunninghamii* and *N*. *moorei* character numbers given in Table 1. Open rectangles represent nonhomoplasious characters including PCR-RFLP, single nucleotide polymorphisms and indels. Black circles represent homoplasious characters, with size of circles indicating consistency index (see key).

Distribution, spatial clustering and regional haplotype diversity

The C1 haplotype was the most frequent (58.2% of samples) and widespread of all haplotypes observed in *N. cunninghamii*. This haplotype occurred across the entire range of the species except the Victorian central highlands, and was almost the only haplotype found in large areas of northwest Tasmania, and populations in the Otway Ranges, Wilsons Promontory, and geographically peripheral populations in Tasmania (Fig. 4a). Haplotype V2 was the second most frequent haplotype (15.0%) and occurred across the central highlands of Victoria. Haplotypes C1 and V2 occurred together in a contact zone of the two haplotypes in the Strzelecki Range (Fig. 4a). Haplotype NE1 (5.1%) was observed only in the northeast highlands of Tasmania (Fig. 4b). The remaining 18 haplotypes were all restricted to western and southern

Tasmania and, apart from WT1 (4.2%), WT5 (2.8%) and WT11 (4.2%), were rare (less than 1.3% of all samples; Fig. 4b,c,d). Five of the rare haplotypes were observed only at high altitudes in inland regions of western and southern Tasmania (WT2 at 900 masl, WT3 at 805 masl, WT6 at 681 masl, WT9 at 610 masl and WT12 at 960 masl). Seven (WT4, WT7, WT10, WT13, WT15, WT17 and WT18) were found only at low altitudes (all below 80 masl) near the coast or inland rivers. High and low altitude haplotypes were scattered throughout the phylogenetic tree.

Across the whole species, the single nearest geographic neighbour analysis detected significant spatial clustering of haplotypes ($P < 0.001$). This may be because significant structuring was evident in Victoria ($P < 0.001$) and in northeast Tasmania ($P < 0.01$). Within western Tasmania the nearest neighbour analysis did not indicate significant clustering ($P > 0.05$). This may be partly due to the presence of C1 haplotype throughout this region, because there was significant clustering ($P = 0.04$) of samples excluding the C1 haplotype. Haplotype diversity was significantly higher in western Tasmania (over twice as high for sample size rarefied to 26 samples) than either northeast Tasmania or Victoria (Table 4). The diversities of the latter two regions were not significantly different. In western Tasmania, a significantly higher ($P < 0.001$; randomisation test) number of haplotypes occurred at low altitudes (12 haplotypes from 43 samples < 150 masl) and at high altitudes (12 haplotypes from 43 samples > 500 masl) than at intermediate altitudes (5 haplotypes from 48 samples, or when rarefied to 43 samples, 4.9 haplotypes).

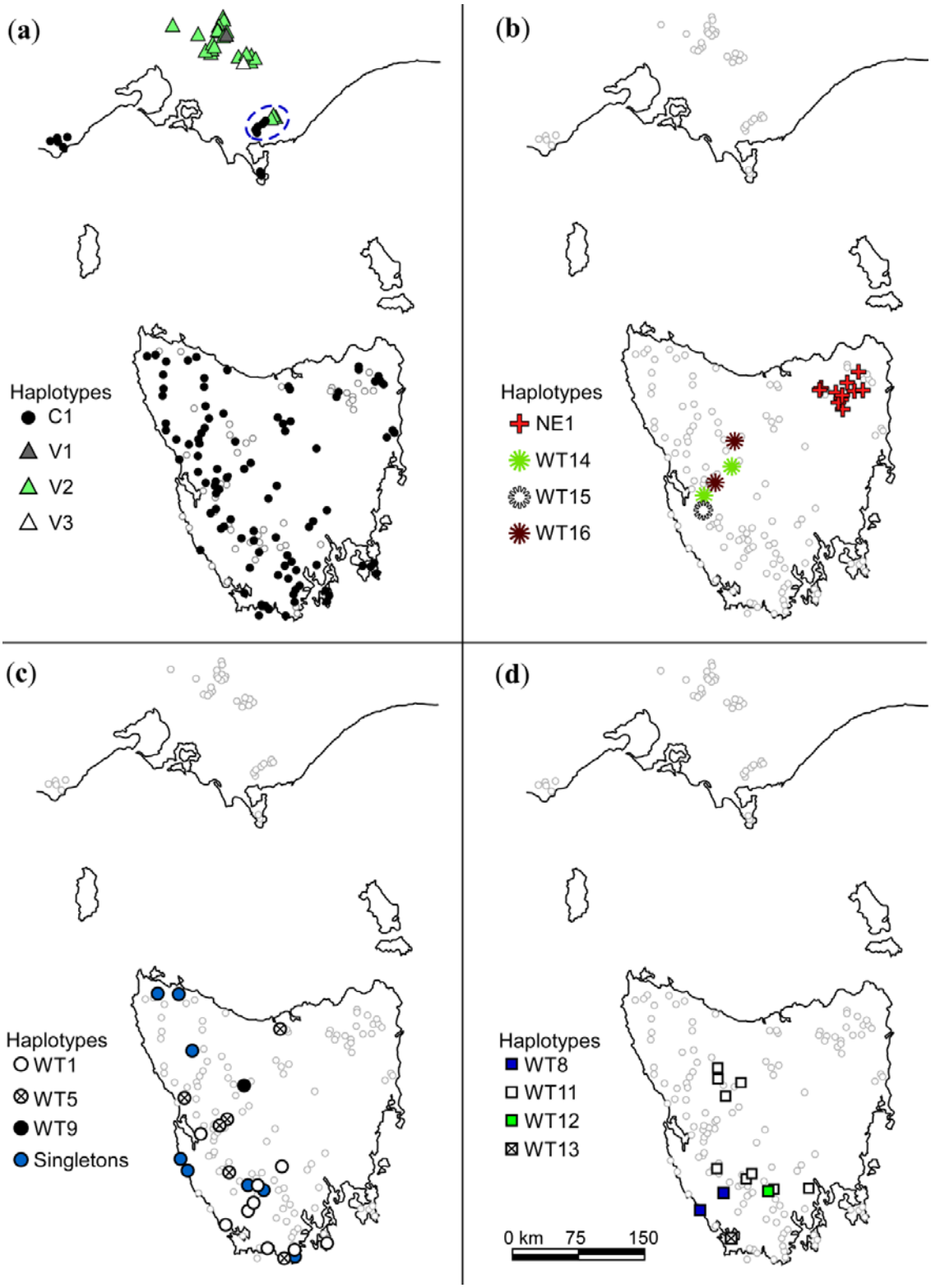


Fig. 4 Distribution of haplotypes observed in *Nothofagus cunninghamii*. For reference, each of the sample locations is displayed on each map as a small open grey circle underlying the haplotypes shown. (a) Distribution of C1 and the minor subclade V1–V3 haplotypes and an inferred contact zone of the C1 and V2 haplotypes in the Strzelecki Range in Victoria (broken circle). (b) Distribution of Clade 2 haplotypes in *N. cunninghamii* (WT14, WT15 and WT16) and the NE1 haplotype. (c) Distribution of haplotypes within subclade A of Clade 1, excluding the C1 haplotype and V1–V3 haplotypes. Singletons are haplotypes that were observed once. (d) Distribution of haplotypes within Clade 1 outside the large subclade A (WT8, WT11, WT12 and WT13).

Table 4 Rarefied haplotype diversity within regions. Values are the mean number of haplotypes when randomly subsampled to 26 samples, ± standard errors. Probabilities of pairwise comparisons of diversity between regions are also given.

Region	Rarefied haplotype diversity (*N*= 26)[a]	Probability[b]	
		NE Tasmania	Victoria
NE Tasmania	2.0 ± 0.0		
Victoria	2.9 ± 0.7	0.161	
W. Tasmania	7.1 ± 1.6	0.001	0.004

[a] Values are the mean number of haplotypes when randomly subsampled to 26 samples, ± SE.

[b] Probability of pairwise comparisons of diversity between regions.

Northeast highlands haplotype fine-scale study

Of a total of 149 *N*. *cunninghamii* individuals sampled from the northeast highlands, 52 carried the widespread C1 haplotype and 97 the NE1 haplotype. A high level of spatial structuring of haplotypes was observed, with two small clusters of the C1 haplotype near the eastern (Blue Tier) and western (Mt Barrow) edges of the region, separated by a large area occupied only by the NE1 haplotype (Fig. 5). The number of individuals with a nearest neighbour bearing the same haplotype was highly non-random ($P < 0.001$). The contact zone between the Mt Barrow cluster and the NE1 haplotype appears to coincide with the course of the St Patricks River. The two haplotypes overlapped only at the southern extreme of the range and at Blue Tier. Both NE1 and C1 haplotypes occurred at altitudes ranging from less than 200 masl to over 1200 masl. Both included large tree and small subalpine shrub forms.

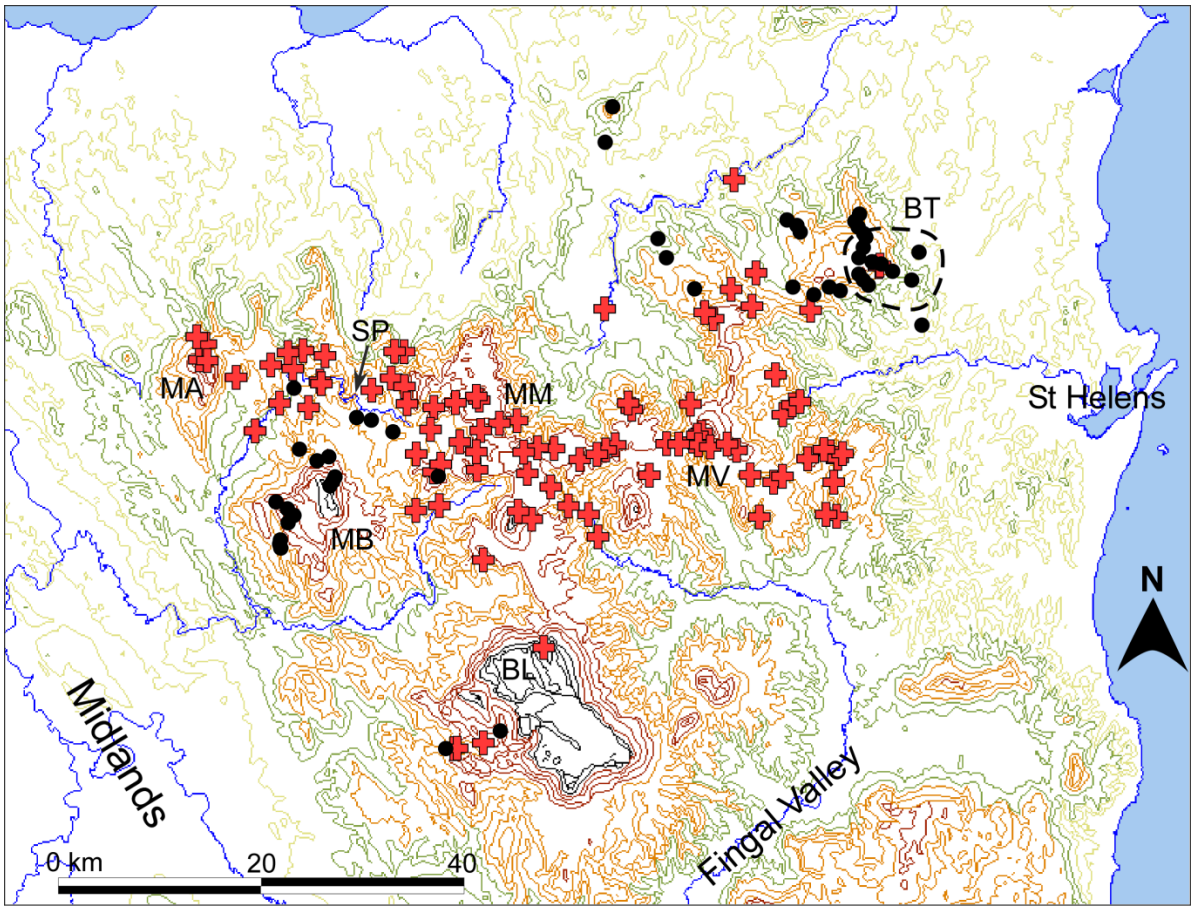


Fig. 5 Distribution of the NE1 (✚) and C1 (●) haplotypes in 149 *Nothofagus. cunninghamii* individuals covering almost the entire species range in the northeast highlands of Tasmania. Features mentioned in the text are Ben Lomond (BL), Blue Tier (BT), Mt Arthur (MA), Mt Barrow (MB), Mt Maurice (MM), Mt Victoria (MV), and the St Patricks River (SP). The black broken circle surrounds the purported location of the Blue Tier glacial refugium from Kirkpatrick & Fowler (1998).

Discussion

Antiquity of haplotypes

Nothofagus cunninghamii was found to harbour remarkably deep and ancient chloroplast divergence. This is strongly indicated by the nesting of the single *N. moorei* haplotype amongst the chloroplast variation found within *N. cunninghamii*. Fossil evidence implies that the split between *N. moorei* and *N. cunninghamii* occurred at least 0.78 million years ago (mya) because this is the minimum age of fossils of both *N. cunninghamii* and an extinct sister species (Jordan & Hill, 1999; Jordan, 1999). It is unlikely that *N. moorei* is a more recent derivative of *N. cunninghamii*, because *N.*

moorei retains plesiomorphic leaf and reproductive traits, whereas *N. cunninghamii* has a range of apomorphic traits (Jordan & Hill, 1999). Indeed, it is likely that the divergence is much older, with estimated molecular dates ranging from 4.2 to 43.6 mya based on chloroplast DNA and 5.1 to 22.4 mya based on ITS sequence data (Knapp *et al.*, 2005; unpublished dates from the analyses of Cook & Crisp 2005; Mike Crisp pers. comm.).

There are other explanations for the nesting of the single *N. moorei* haplotype within haplotype variation observed in *N. cunninghamii*. One hypothesis is transfer of chloroplast DNA through direct hybridisation between *N*. *cunninghamii* and *N*. *moorei*. Plant species can capture the maternally inherited chloroplast of other species through repeated backcrossing of inter-specific hybrids (as shown in *Quercus* (Dumolin-Lapegue et al., 1997a) and *Eucalyptus* (McKinnon *et al.*, 2004). However, recent chloroplast capture is unlikely in the present case because *N. moorei* and *N. cunninghamii* are now separated by ~ 780 km, and *N. moorei* has a single haplotype across a 500 km range. In contrast, ancient chloroplast capture (either with *N. moorei* or using an extinct species as a bridge) is possible. Extinct relatives of *N. cunninghamii* and *N. moorei* are known to have occurred in Tasmania up to the Early Pleistocene (Hill & Read, 1991; Jordan, 1999). The likelihood of ancient hybridisation is heightened by the fact that barriers to hybridisation appear low within *Nothofagus* subgenus *Lophozonia*. Thus, spontaneous hybrids occur in cultivation between New Zealand and Chilean species (Wingston, 1979), and natural hybridisation between species is common in Chile and Argentina (Donoso & Landrum, 1979; Marchelli & Gallo, 2001, 2004). A final hypothesis is lineage sorting, in which the gene pool of *N*. *cunninghamii* has retained several disparate ancestral cpDNA lineages whose divergence predated speciation. If correct this interpretation would indicate that divergence of Clades 1 and 2 and haplotype NE1 is likely to be millions of years old.

Western Tasmania: the major reservoir of chloroplast diversity

Western Tasmania is the stronghold of haplotype diversity in *N*. *cunninghamii* with eighteen endemic haplotypes including three Clade 2 haplotypes (WT14, WT15 and WT16) that demonstrate considerable divergence from the commonest haplotype (C1). The rarefaction analyses show clearly that this high diversity is not an artefact of more intensive sampling in this region. The antiquity of haplotypes and high haplotype diversity within western Tasmania are both consistent with a long history

of *N. cunninghamii* within the region. Similar interpretations of long term occupation of an area based on high chloroplast diversity and ancestral variation have been made in a three *Quercus* spp. complex in the western Mediterranean basin (Jiminez *et al.*, 2004) and *Lithocarpus* spp. in Southeast Asia (Cannon & Manos, 2003). This is the first genetic evidence that demonstrates the importance of western Tasmania as a reservoir of genetic diversity for cool temperate rainforest species in southeastern Australia, a role that has been postulated based on the restriction of many paleo-endemic (e.g. *Athrotaxis* spp., *Lagarostrobos franklinii*, *N. gunnii*, *Microcachrys tetragona* and *Diselma archeri*) cool temperate rainforest plant species to this region (Colhoun, 1985a).

Molecular evidence for glacial refugia outside regions of expected survival

The complex patchy distribution of haplotypes of *Nothofagus cunninghamii* across its range strongly supports the concept of multiple glacial rainforest refugia. The evidence for this comes from many parts of the species' range containing endemic haplotypes combined with evidence of antiquity of the haplotypes. In terms of the location of glacial refugia, the high haplotype diversity at low altitudes in western Tasmania (including seven rare haplotypes observed only below 80 masl) is remarkably consistent with estimates of the LGM tree line (Colhoun, 1985a; Gibson *et al.*, 1987) where survival of cool temperate rainforest species is thought to have occurred (Colhoun, 2000). However, the significant number of haplotypes restricted to high altitudes (a total of five; including three haplotypes observed only above 800 masl), suggests that *N. cunninghamii* may also have survived well above the estimated LGM tree line, but below the permafrost zone. The high diversity of haplotypes at high altitudes is unlikely to be a result of a 'phalanx' type postglacial expansion, whereby a high diversity of haplotypes can be retained during migration (Hewitt, 1996), because five of the high altitude haplotypes were not found at low or mid-altitudes. Evidence for high altitude refugia was unexpected because *N. cunninghamii* currently has a very limited occurrence above the climatic tree line (Macphail, 1975; Harle *et al.*, 1993; Kirkpatrick & Fowler, 1998).

The restriction of haplotype NE1, which fell outside both Clades 1 and 2, to the northeast highlands of Tasmania (Fig. 2) is stark evidence for glacial survival of *N. cunninghamii* within this region. Considering that the divergence of this haplotype from other *N. cunninghamii* haplotypes is at least as old as the divergence between *N.*

cunninghamii and *N*. *moorei* haplotypes, *N*. *cunninghamii* has plausibly survived through multiple glacial/ interglacial cycles of the Pleistocene in the northeast of Tasmania. An explanation that does not require glacial survival must involve Holocene dispersal from a population outside the northeast. However this scenario is unlikely since neither the NE1 haplotype, nor any related haplotype, was observed in 187 samples covering nearly the entire species' range in western Tasmania and Victoria, including all populations close to the northeast highlands. We infer therefore that glacial refugia harbouring the NE1 haplotype occurred in the vicinity of some of the major mountain peaks (e.g. Mt Victoria, Mt Maurice and Mt Arthur), or in lowland riparian habitats, within the haplotype's current distribution. This is the first genetic evidence of glacial refugia for cool temperate rainforest species in the northeast highlands. This finding fits well with the occurrence of a distinctive invertebrate fauna that includes some slow dispersing centipede, millipede, snail, and beetle species endemic to the region (Mesibov, 1994, 1997; Munks *et al.*, 2004).

The strong spatial structuring of the C1 and NE1 haplotypes observed in the northeast Tasmanian highlands suggests at least two and plausibly more refugia within the northeast. The two C1 haplotype patches near the eastern and western extremes of the distribution of *N*. *cunninghamii* in the northeast (Mt Barrow and Blue Tier) may best be explained as being derived from postglacial expansion from populations that survived the LGM *in situ* within these two areas. It is unlikely that two long-distance seed dispersal events from western Tasmanian refugia were able to found both these patches during the Holocene. A long-distance seed dispersal explanation is especially unlikely considering these events would have had to occur in a narrow window before individuals harbouring the NE1 haplotype were able to expand into these two areas.

Chloroplast DNA evidence for multiple refugia within northeast Tasmania is hard to reconcile with the strong evidence that the eastern Tasmanian climate was drier than present during the LGM. The evidence for this comes from widespread occurrence of arid formed sand dunes in lowland parts (Bowden, 1983; Colhoun, 2002; Duller & Augustinus, 2006) and the confinement of glaciation to one very small part of the southeastern flank of the Ben Lomond plateau (Barrows *et al.*, 2002; Fig. 5) despite these mountains being as high as the heavily glaciated mountains of western Tasmania (Derbyshire, 1966; Colhoun & Fitzsimons, 1990). In particular, it is difficult

to reconcile arid formed sand-dunes with sufficient growing season rainfall required to sustain *N. cunninghamii*. Even by forcing their palaeoclimatic model to have relatively wet climates in eastern Tasmania during the LGM, Kirkpatrick & Fowler (1998) could not infer climates suitable for *N. cunninghamii* in the western and central parts of the northeast (i.e. outside of Blue Tier, BT; Fig. 5), areas which our data suggests should have contained refugia.

One possible explanation for survival in such dry environments would be that the past tolerance of the species was greater than would be predicted from its current distribution. This hypothesis is reinforced by the high diversity of haplotypes found at high altitudes in western Tasmania - at altitudes more than 300 m above the estimated last glacial treeline. The ability of *N. cunninghamii* to coppice from basal buds after desiccation of the main stem (Howard, 1973) may have contributed to the persistence of this species through glacial periods, although it appears unlikely that individuals could have survived by this mechanism through the full 8,000 year span of the peak of the last glacial. This would mean that all life stages would have been exposed to full glacial climates.

The widespread subclade and Victorian populations

Although subclade A contained 86% of the samples in the broad-range survey and occurred across the entire range of *N. cunninghamii*, it is a relatively derived clade (Fig. 3) and at least three, and plausibly five or more haplotype lineages were present at the time that subclade A evolved. Given that these other lineages are now very restricted in spatial range and mostly uncommon, this would suggest that individuals carrying haplotypes from subclade A expanded to cover the entire range of the species, while other haplotypes present at that time were displaced or failed to expand. Trees carrying haplotypes of subclade A may have had an adaptive advantage over other clades. This expansion is likely to predate the last glacial given the number of derived haplotypes in this clade. Although the diversity of subclade A haplotypes is highest in western Tasmania which suggests that this region is likely the origin of this clade, the presence of the *N. moorei* haplotype (now geographically located more than 1000 km to the north of Tasmania) nested within the *N. cunninghamii* lineage shows that the ancient history of the subgenus in southeastern Australia is complex.

The phylogeny (Fig. 3) further suggests that the C1 haplotype is likely to be the ancestral haplotype for subclade A because all the other haplotypes within this subclade can most parsimoniously be derived from it. Thus, the widespread distribution of C1 is not evidence for Holocene migration. While Holocene migration may be a possible explanation, the presence of the C1 haplotype in southern Victoria may equally represent an earlier expansion of subclade A. The presence of fossil leaves and fruit of *N. cunninghamii* on King Island in the Last Interglacial (G.J. Jordan unpublished) would suggest a potential corridor for this expansion (Fig. 1).

The central highland Victorian subclade V1-V3 corroborates the pollen evidence from within this region for *in situ* LGM survival of *N. cunninghamii* (McKenzie, 1997). An alternative scenario whereby these haplotypes arose after Holocene migration into the region is unlikely because of the presence of three related, endemic haplotypes and the complete absence of the C1 haplotype that is elsewhere so widespread.

The haplotype contact zone in the Strzelecki Ranges (Fig. 4a) may represent recent migration of the C1 and/or V2 haplotypes into this region. Haplotype contact zones in *Fagus crenata* in central Japan (Kobashi *et al.*, 2006) and *Quercus robur* in southern Finland (Ferris *et al.*, 1998) have been interpreted as Holocene events. However, whether the 'islands' of cool temperate rainforest in the Otway Ranges and Wilsons Promontory are derived from glacial refugia contained within these two regions is uncertain, a question that may be addressed by phylogeographic studies of more widespread rainforest species that occur in one or both of the Otway Ranges and Wilsons Promontory, such as *Acmena smithii* (Myrtaceae), *Atherosperma moschatum* (Atherospermataceae), *Elaeocarpus reticulatus* (Elaeocarpaceae), *Hedycarya angustifolia* (Monimiaceae), *Lomatia fraseri* (Proteaceae), *Pittosporum bicolor* (Pittosporaceae) and *Tasmannia lanceolata* (Winteraceae).

Conclusions

The complex patchy distribution of haplotypes provides strong evidence that most current populations are derived from short-range dispersal probably less than 100 km from nearby glacial refugia. This contrasts with the continent wide postglacial movements apparent in some forest trees in Europe (Demesure *et al.*, 1996; Dumolin-Lapegue *et al.*, 1997a). Considering the antiquity of haplotypes in *N. cunninghamii*, patterns of genetic variation almost certainly significantly predate the LGM a finding that adds to the increasing literature on the ever deeper phylogeographies present in

current populations of forest trees (Lumaret *et al.*, 2002; Grivet *et al.*, 2006; Hampe & Petit, 2007; Magri *et al.*, 2007). This study provides the first genetic evidence of the importance of the topographically diverse western half of Tasmania in providing long term buffered climates for rainforest species throughout the Pleistocene. Interestingly, the chloroplast evidence indicates that *N*. *cunninghamii* was able to survive in regions that were unexpected based on our knowledge of glacial climates in southeastern Australia. The most extreme case appears to be the strong evidence for multiple refugia of *N*. *cunninghamii* within the northeast highlands, a finding that adds to the growing body of evidence for temperate tree survival in non-equable climates beyond the understood physiological tolerances of species during the LGM and further demonstrates the important role that mountainous regions have in providing refugia though climatic changes (Hewitt, 2000; Hewitt, 2004).

Appendices

Appendix 1

Nothofagus cunninghamii sample information for the range-wide chloroplast study (213 samples), including sample information for the five *N. moorei* samples. The haplotype determined for each sample is shown.

N	Stand	Region	Location Name	Latitude	Longitude	Alt.	Haplotype
1	1	W. Tasmania	Lake Dobson Road, Mt Field NP	-42.67978	146.69949	350	WT1
2	2	NE Tasmania	St Patricks River	-41.33317	147.34070	360	NE1
3	3	NE Tasmania	Myrtle Bank Road	-41.27995	147.35800	500	NE1
4	4	NE Tasmania	Diddleum Plains	-41.32418	147.45171	590	C1
5	5	NE Tasmania	Ben Ridge Road	-41.36837	147.64244	860	NE1
6	6	NE Tasmania	upper St Patricks River	-41.34373	147.56699	640	NE1
7	7	NE Tasmania	Mount Barrow	-41.37875	147.42305	1200	C1
8	8	NE Tasmania	Ben Lomond, Strickland Corner	-41.51311	147.66066	1340	NE1
9	9	NE Tasmania	Telopea Road	-41.40642	147.64677	890	NE1
10	10	NE Tasmania	Billybrook Creek, Traills Point	-41.44073	147.59401	680	NE1
11	11	W. Tasmania	Mt Read	-41.84602	145.54137	1136	C1
12	12	W. Tasmania	Ibsens Peak nr Lake Gordon	-42.79903	146.35492	530	WT11
13	13	W. Tasmania	Sentinel Range	-42.87036	146.23849	900	WT2
14	14	W. Tasmania	Scotts Peak Dam	-43.04006	146.30145	300	WT1
15	15	W. Tasmania	Serpentine Creek	-42.86934	146.36705	360	WT1
16	16	W. Tasmania	Condominium Creek	-42.95759	146.36615	320	C1
17	17	W. Tasmania	Millers Bluff	-41.91499	147.16113	1090	C1
18	18	W. Tasmania	Mt Wellington, Big Bend	-42.88798	147.22119	1100	WT11
19	19	W. Tasmania	Clear Hill Road	-42.74678	146.29037	340	C1
20	20	W. Tasmania	Gordon River Road, Stillwater Picnic Area	-42.82376	146.10918	320	C1
21	21	W. Tasmania	Timbs Track nr Little Florentine River	-42.73936	146.42094	520	WT11
22	22	W. Tasmania	Gordon River, near Gordon Dam	-42.74709	145.96448	110	WT5
23	23	W. Tasmania	Mt Black	-41.75409	145.56003	661	C1
24	24	W. Tasmania	Wayatinah	-42.41981	146.48621	261	C1
25	25	W. Tasmania	Lake St Clair	-42.11320	146.16202	720	WT14
26	25	W. Tasmania	Lake St Clair	-42.11544	146.16411	720	WT14
27	25	W. Tasmania	Lake St Clair	-42.11337	146.16257	720	C1
28	26	W. Tasmania	Double Barrel Creek	-42.19451	145.94640	435	WT5
29	27	W. Tasmania	Snake Creek	-42.11677	145.78682	505	C1
30	28	W. Tasmania	Mount Jukes Road	-42.15104	145.52699	96	C1
31	29	W. Tasmania	Bird River	-42.34459	145.58914	93	WT1
32	30	W. Tasmania	Crotty River	-42.25278	145.61766	200	C1
33	31	W. Tasmania	Henty Bridge, Henty River	-42.02409	145.26227	254	C1
34	32	W. Tasmania	Chevron Creek nr Zeehan	-41.97356	145.36185	159	WT5
35	33	W. Tasmania	Heemskirk River	-41.81506	145.21587	176	C1
36	34	W. Tasmania	Corrina	-41.71867	145.07390	210	C1
37	35	W. Tasmania	Pieman River	-41.65443	145.07440	18	C1
38	36	W. Tasmania	Mt Read	-41.81226	145.52701	637	C1
39	37	W. Tasmania	Murchison Highway- nr Station Creek	-41.87286	145.39330	216	C1
40	38	W. Tasmania	Henty Glacial Moraine	-41.98855	145.51317	396	C1
41	39	W. Tasmania	Tramway Creek near Tullah	-41.69020	145.60216	289	C1
42	40	W. Tasmania	Bulgobac River	-41.60331	145.66959	660	C1
43	41	W. Tasmania	Ramsay River near Luina	-41.48687	145.46497	681	WT6
44	42	W. Tasmania	Mt Pearse	-41.47175	145.62679	652	C1
45	43	W. Tasmania	Vale River near Rocky Mount	-41.54533	145.86707	887	C1
46	44	W. Tasmania	Lake Dove	-41.65539	145.96278	974	WT11

47	45	W. Tasmania	Mt Fortescue, Tasman Peninsula	-43.17229	147.96579	380	C1
48	46	W. Tasmania	Coolangatta Road, Bruny Island	-43.35835	147.28630	440	C1
49	47	W. Tasmania	Captain Cook Creek, Bruny Island	-43.40004	147.31923	175	C1
50	48	W. Tasmania	Sheepwash Creek, Bruny Island	-43.45961	147.30162	340	C1
51	49	W. Tasmania	Simmonds Creek, Tasman Peninsula	-43.11517	147.90343	170	C1
52	50	W. Tasmania	Tatnells Hill, Tasman Peninsula	-43.07250	147.94580	480	C1
53	51	W. Tasmania	Frenchmans Cap	-42.26669	145.82990	1200	WT5
54	52	W. Tasmania	Philps Creek	-42.28056	145.91235	430	WT16
55	53	W. Tasmania	Russell River	-42.90184	146.72990	360	WT11
56	54	W. Tasmania	Denison Road	-42.98982	146.74876	340	C1
57	55	W. Tasmania	Huon River	-43.05890	146.81667	80	C1
58	56	W. Tasmania	Mt Picton Track	-43.19177	146.62920	485	C1
59	57	W. Tasmania	Hartz Mountains	-43.22873	146.76430	910	C1
60	58	W. Tasmania	Nevada Peak Track	-42.92047	146.66023	960	WT12
61	59	W. Tasmania	Esperance River	-43.30420	146.92110	80	C1
62	60	W. Tasmania	Tahune Forest Reserve	-43.09764	146.72581	70	C1
63	61	W. Tasmania	Arve River Road	-43.14379	146.87616	320	C1
64	62	W. Tasmania	Emu River	-41.09357	145.92657	70	C1
65	63	Victoria	Maits Rest, Otway Ranges	-38.73706	143.65045	272	C1
66	64	Victoria	Melba Gully State Park, Otway Ranges	-38.67026	143.47183	368	C1
67	64	Victoria	Melba Gully State Park, Otway Ranges	-38.66998	143.47093	374	C1
68	65	Victoria	Deppler Creek, Otway Ranges	-38.63559	143.71095	379	C1
69	66	Victoria	Wait-a-While Road, Otway Ranges	-38.64117	143.55757	541	C1
70	67	Victoria	Aire River, Otway Ranges	-38.68092	143.57761	187	C1
71	68	Victoria	Kingslake NP, Central Highlands	-37.43354	145.16463	633	V2
72	69	Victoria	Wirra-Willa, Central Highlands	-37.53026	145.51816	617	V2
73	70	Victoria	Yarra Ranges NP, Central Highlands	-37.71531	145.62022	644	V2
74	71	Victoria	Mt Donna Buang, Central Highlands	-37.71332	145.64767	644	V2
75	72	Victoria	Mt Donna Buang, Central Highlands	-37.70898	145.68053	1257	V2
76	73	Victoria	Yarra Ranges NP, Central Highlands	-37.72935	145.69637	935	V2
77	74	Victoria	Yarra Ranges NP, Central Highlands	-37.70956	145.70831	909	V2
78	75	Victoria	Yarra Ranges NP, Central Highlands	-37.69261	145.71741	680	V2
79	76	Victoria	Yarra Ranges NP, Central Highlands	-37.66961	145.74081	714	V2
80	77	Victoria	Yarra Ranges NP, Central Highlands	-37.64320	145.71964	657	V2
81	78	Victoria	Cumberland Road, Central Highlands	-37.53114	145.82043	964	V2
82	79	Victoria	Cumberland Road, Central Highlands	-37.55914	145.86070	998	V2
83	80	Victoria	Cumberland Road, Central Highlands	-37.54827	145.89255	999	V2
84	81	Victoria	Cumberland Road, Central Highlands	-37.53282	145.90141	983	V2
85	82	Victoria	Camberville Road, Central Highlands	-37.51913	145.91139	983	V1
86	83	Victoria	Lady Talbot Road, Central Highlands	-37.50240	145.79836	436	V2
87	84	Victoria	Lady Talbot Road, Central Highlands	-37.49952	145.84702	436	V2
88	85	Victoria	Lady Talbot Road, Central Highlands	-37.47267	145.82111	852	V2
89	86	Victoria	Blue Range Road, Central Highlands	-37.44173	145.80536	268	V2
90	87	W. Tasmania	Styx River	-42.81753	146.59938	380	C1
91	88	Victoria	Royston River Road, Central Highlands	-37.34792	145.86577	222	V2
92	89	Victoria	Royston River Road, Central Highlands	-37.38532	145.88443	240	V2
93	90	Victoria	Thompson Valley, Central Highlands	-37.79754	146.32654	967	V2
94	91	Victoria	Thompson Valley, Central Highlands	-37.78241	146.28770	1069	V2
95	92	Victoria	Thompson Valley, Central Highlands	-37.75729	146.26752	1084	V2
96	93	Victoria	Thompson Valley, Central Highlands	-37.75011	146.20348	1123	V2
97	94	Victoria	Noojee, Central Highlands	-37.78544	146.07132	797	V2
98	95	Victoria	Tarra Bulga NP, Strzelecki Range	-38.43757	146.57608	594	V2
99	96	Victoria	Tarra Bulga NP, Strzelecki Range	-38.42234	146.56987	668	V2
100	97	Victoria	Tarra Bulga NP, Strzelecki Range	-38.45648	146.54352	265	V2
101	98	Victoria	Jeeralang Creek, Strzelecki Range	-38.46701	146.44798	476	C1
102	99	Victoria	Sealers Cove, Wilsons Promontory	-39.02921	146.38521	10	C1

103	100	W. Tasmania	Projection Bluff	-41.72517	146.72107	1240	C1
104	101	W. Tasmania	Dee River	-42.28912	146.61307	650	C1
105	102	NE Tasmania	upper Apsley River, Douglas-Apsley	-41.79671	148.12302	504	C1
106	102	NE Tasmania	upper Apsley River, Douglas-Apsley	-41.79665	148.12300	525	C1
107	103	NE Tasmania	Lookout Hill, Douglas-Apsley	-41.73850	148.22604	476	C1
108	103	NE Tasmania	Lookout Hill, Douglas-Apsley	-41.73850	148.22571	476	C1
109	103	NE Tasmania	Lookout Hill, Douglas-Apsley	-41.73811	148.22678	472	C1
110	104	NE Tasmania	Douglas River, Douglas-Apsley	-41.73297	148.20537	404	C1
111	105	NE Tasmania	Ransom River	-41.24854	148.07495	105	C1
112	106	NE Tasmania	Poimena Road	-41.21463	148.01453	579	C1
113	107	NE Tasmania	Blue Tier	-41.16082	148.00212	662	C1
114	108	NE Tasmania	Blue Tier	-41.15986	148.00313	683	C1
115	109	NE Tasmania	Weldborough Pass	-41.22216	147.95500	570	C1
116	110	NE Tasmania	Ringarooma River nr Moorina	-41.12634	147.86852	118	NE1
117	111	NE Tasmania	Mt Horror	-41.06694	147.73257	670	C1
118	112	NE Tasmania	Mt Horror Road	-41.09626	147.72505	220	C1
119	113	NE Tasmania	Ringarooma River	-41.23336	147.72609	205	NE1
120	114	NE Tasmania	South George River	-41.31242	147.93417	261	NE1
121	115	NE Tasmania	upper Ringarooma River	-41.31255	147.81975	700	NE1
122	116	W. Tasmania	Long Ridge	-41.58951	146.62842	263	C1
123	117	W. Tasmania	Cluan Tier	-41.63508	146.80136	722	C1
124	118	W. Tasmania	Sumac Spur 2	-41.14323	145.03923	170	C1
125	119	W. Tasmania	Yarlington Tier	-42.53892	147.29938	670	C1
126	120	W. Tasmania	Lake Windermere	-41.76754	145.94969	1005	WT11
127	121	W. Tasmania	Pelion Plains	-41.83130	146.03055	835	C1
128	122	W. Tasmania	Pelion Creek	-41.83413	146.04628	950	C1
129	123	W. Tasmania	Pine Valley	-41.95855	146.06120	843	WT11
130	124	W. Tasmania	Herods Gate	-41.80925	146.27827	1180	WT11
131	125	W. Tasmania	Jacksons Creek	-41.85136	146.17722	610	WT9
132	125	W. Tasmania	Jacksons Creek	-41.85136	146.17722	610	WT16
133	125	W. Tasmania	Jacksons Creek	-41.85136	146.17722	610	WT9
134	126	W. Tasmania	Andersons Creek, Dazzler Ranges	-41.26631	146.76660	260	C1
135	127	W. Tasmania	Andersons Creek, Dazzler Ranges	-41.29023	146.77608	315	C1
136	128	W. Tasmania	Saxons Creek, Dazzler Ranges	-41.27175	146.66629	112	WT5
137	129	W. Tasmania	Evans Creek	-42.60281	145.30554	40	WT4
138	130	W. Tasmania	Gordon River	-42.73066	145.84644	35	C1
139	131	W. Tasmania	Denison River	-42.63144	145.97217	152	C1
140	132	W. Tasmania	Denison River	-42.70097	145.86839	60	C1
141	133	W. Tasmania	Franklin River	-42.52954	145.77342	35	C1
142	134	W. Tasmania	Franklin River	-42.54901	145.75270	30	WT15
143	135	W. Tasmania	Celery Top Islands	-43.37359	146.15445	8	C1
144	136	W. Tasmania	Claytons, Forest Lag	-43.37376	146.12852	10	WT13
145	137	W. Tasmania	Celery Top Islands	-43.37687	146.15874	11	C1
146	138	W. Tasmania	Capella Crags, Arthur Range	-43.12535	146.23105	890	C1
147	139	W. Tasmania	Mount Hesperus, Arthur Range	-43.11362	146.22527	870	WT1
148	140	W. Tasmania	Adamsons Peak Track	-43.33968	146.86763	310	C1
149	141	W. Tasmania	De Witt Island	-43.59283	146.35977	54	C1
150	142	W. Tasmania	Deadmans Creek	-43.52064	146.51025	10	WT1
151	143	W. Tasmania	Mt Clark, Tasman Peninsula	-43.10061	147.78181	445	C1
152	144	W. Tasmania	Platform Peak	-42.70383	147.06348	680	C1
153	145	W. Tasmania	Mt Banks, Bruny Island	-43.43706	147.31633	360	WT1
154	146	W. Tasmania	Detention River	-40.96099	145.48889	103	C1
155	147	W. Tasmania	Doughboy Hill	-40.99328	145.37354	134	C1
156	148	W. Tasmania	Black River	-40.90307	145.28540	74	WT10
157	149	W. Tasmania	Lindsay River	-41.36407	145.02904	278	C1
158	150	W. Tasmania	Sumac Road	-41.28928	145.04063	71	C1

159	151	W. Tasmania	Rattler River	-41.17990	145.60339	380	C1
160	152	W. Tasmania	Farnhams Creek	-40.90562	144.97643	60	WT7
161	153	W. Tasmania	Julius River Forest Reserve	-41.15576	145.02634	80	C1
162	154	W. Tasmania	Riseborough Road	-40.94275	144.92920	82	C1
163	155	W. Tasmania	Hays Tier	-40.95335	144.81264	91	C1
164	156	W. Tasmania	Hellyer Gorge State Reserve	-41.27446	145.61270	273	C1
165	157	W. Tasmania	Roger River	-41.01427	145.06396	62	C1
166	158	W. Tasmania	Snug Tiers	-43.09048	147.16034	640	C1
167	159	W. Tasmania	Travellers Rest Lake	-42.05191	146.23869	960	C1
168	160	W. Tasmania	Sanctuary Lake, Frankland Range	-42.93270	146.03052	680	WT8
169	161	W. Tasmania	Mt Anne	-42.92860	146.42833	805	WT3
170	162	W. Tasmania	Rossilyns Pool, Denison River	-42.68156	145.93814	400	WT11
171	163	W. Tasmania	Gordon River	-42.71819	145.83449	61	C1
172	164	W. Tasmania	South Cape Range	-43.59872	146.73009	457	C1
173	164	W. Tasmania	South Cape Range	-43.59870	146.73007	458	C1
174	164	W. Tasmania	South Cape Range	-43.59858	146.73021	463	WT5
175	165	W. Tasmania	Ironbound Range	-43.50543	146.45308	832	C1
176	166	W. Tasmania	Louisa River	-43.48703	146.41023	62	C1
177	167	W. Tasmania	Little Deadmans Bay	-43.53040	146.49651	1	C1
178	167	W. Tasmania	Little Deadmans Bay	-43.53026	146.49660	1	C1
179	168	W. Tasmania	Mt Dromedary	-42.65580	147.11542	940	C1
180	169	Victoria	Mt Ramsey, Wilsons Promontory	-39.02664	146.38290	560	C1
181	170	Victoria	Mt Ramsey, Wilsons Promontory	-39.01975	146.37968	647	C1
182	171	Victoria	Mt Latrobe, Wilsons Promontory	-39.00454	146.37700	733	C1
183	172	Victoria	Mt Latrobe, Wilsons Promontory	-39.00237	146.37749	786	C1
184	173	Victoria	trib. of Tidal River, Wilsons Promontory	-38.99970	146.36639	519	C1
185	174	Victoria	w. side of Mt Latrobe, Wilsons Promontory	-39.00100	146.37222	649	C1
186	175	Victoria	tributory of Falls Creek, Strzelecki Range	-38.58517	146.31152	294	C1
187	176	Victoria	headwaters of Agnes River, Strzelecki Range	-38.55581	146.31464	448	C1
188	177	Victoria	headwaters of Agnes River, Strzelecki Range	-38.54145	146.31773	433	C1
189	178	Victoria	The Grand Ridge Road, Strzelecki Range	-38.53390	146.32518	491	C1
190	179	Victoria	Albert River, Strzelecki Range	-38.50666	146.39668	323	C1
191	180	Victoria	Tarra Bulga NP, Strzelecki Range	-38.42728	146.57130	592	V2
192	181	Victoria	tributory of Tanjil River, Central Highlands	-37.84295	146.15485	640	V2
193	182	Victoria	East Tanjil Road,, Central Highlands	-37.83202	146.18912	514	V3
194	183	Victoria	Baw Baw Alpine Village, Central Highlands	-37.83752	146.25793	1466	V2
195	184	Victoria	Lake Mountain summit, Central Highlands	-37.49758	145.87706	1396	V2
196	185	W. Tasmania	Quamby Bluff summit	-41.65398	146.69557	1193	C1
197	186	W. Tasmania	Mt Wedge	-42.84538	146.29196	960	C1
198	187	W. Tasmania	Christmas Cove	-42.72397	145.39154	10	WT18
199	188	W. Tasmania	Lewis River	-42.94516	145.55208	41	C1
200	189	W. Tasmania	Hastings Caves	-43.38297	146.84111	148	C1
201	190	W. Tasmania	South Lune Road	-43.46359	146.85805	82	C1
202	191	W. Tasmania	D'Entrecasteaux River	-43.50801	146.87451	21	WT1
203	192	W. Tasmania	Cockle Creek	-43.57571	146.88684	19	WT17
204	193	W. Tasmania	confluence of Collingwood and Franklin Rvs	-42.20039	145.93115	308	C1
205	194	W. Tasmania	Angel Rain, Franklin River	-42.21815	145.90649	316	C1
206	195	W. Tasmania	Finchams Crossing, Franklin River	-42.24186	145.76730	219	C1
207	196	W. Tasmania	Camp Arcade, Franklin River	-42.28542	145.74736	231	C1
208	197	W. Tasmania	Coruscades, Franklin River	-42.32933	145.79338	181	C1
209	198	W. Tasmania	Rafters Basin	-42.36467	145.77173	113	C1
210	199	W. Tasmania	Newlands Cascade	-42.42163	145.75577	124	WT14
211	200	W. Tasmania	Sir John Falls	-42.56985	145.68976	21	C1
212	201	W. Tasmania	Whalers Cove	-43.26947	145.92546	7	WT1
213	202	W. Tasmania	Mulcahy River	-43.10702	145.71570	22	WT8

1	*N. moorei*	NSW/ SE Qld	Honeysuckle Rest Area Barrington Tops	-31.90445	151.53538	1340	Nm
2	*N. moorei*	NSW/ SE Qld	Gibsons Mill, Barrington Tops	-31.90642	151.63378	1180	Nm
3	*N. moorei*	NSW/ SE Qld	Tullawallal Lookout (Lamington NP)	-28.21111	153.19056	935	Nm
4	*N. moorei*	NSW/ SE Qld	Springbrook NP	-28.24167	153.26500	1000	Nm
5	*N. moorei*	NSW/ SE Qld	The Bar Mountain	-28.46028	153.13472	1095	Nm

Appendix 2

Sample information for the northeast highlands haplotype fine-scale study (129 samples). The haplotype determined for each sample is shown.

N	Stand	Location	Latitude	Longitude	Alt.	Haplotype
214	203	Nile River	-41.59721	147.55928	587	C1
215	203	Nile River	-41.59715	147.56067	593	NE1
216	204	Nile River	-41.59718	147.56203	597	NE1
217	205	Doaks Road near Mt Arthur	-41.25623	147.27689	397	NE1
218	206	Doaks Road near Mt Arthur	-41.26241	147.28658	598	NE1
219	207	tributory of Patersonia Rivulet	-41.29117	147.31935	448	NE1
220	208	Bennies Creek	-41.34865	147.38916	530	C1
221	209	Seven Time Creek	-41.31469	147.40042	458	NE1
222	210	St Patricks River	-41.29721	147.41562	440	NE1
223	211	St Patricks River	-41.29332	147.41341	437	NE1
224	212	Beckett Creek	-41.40042	147.51859	371	NE1
225	213	Northallerton Road	-41.39655	147.54460	510	NE1
226	214	Sunset Ridge	-41.37286	147.53873	902	C1
227	215	Sunset Ridge	-41.36707	147.53845	924	NE1
228	216	Ben Ridge Road	-41.36132	147.54700	795	NE1
229	217	Ben Ridge Road	-41.36631	147.58681	925	NE1
230	218	Upper Esk Road	-41.37904	147.66809	811	NE1
231	219	Memory Creek	-41.40306	147.70818	426	NE1
232	220	Upper South Esk River	-41.42142	147.71753	393	NE1
233	221	Poimena Road	-41.21027	148.00980	651	C1
234	222	Tombstone Creek Forest Reserve	-41.39534	147.68707	601	NE1
235	223	Poimena Road	-41.20533	148.00465	732	C1
236	224	Sun Flats Road	-41.19187	148.00523	750	C1
237	225	Mount Michael	-41.18346	148.00972	748	C1
238	226	Blue Tier	-41.17426	148.01112	755	C1
239	227	Blue Tier	-41.16856	148.00728	684	C1
240	228	Sun Flats	-41.19618	148.01901	755	C1
241	229	Sun Flats Road	-41.19947	148.02963	666	NE1
242	230	Sun Flats Road	-41.20288	148.04294	523	C1
243	231	Great Musselroe River	-41.18701	148.07178	202	C1
244	232	Swan Rivulet	-41.21063	148.06375	320	C1
245	233	Crystal Hill	-41.21894	147.98398	533	C1
246	234	Groom River	-41.21611	147.97182	553	C1
247	235	Weldborough Pass	-41.21634	147.93257	462	C1
248	236	Paris Dam Road	-41.20319	147.89346	502	NE1
249	237	Minnie Jessop Picnic Ground	-41.21723	147.86513	551	NE1
250	238	Britannia Creek	-41.21722	147.82368	666	C1
251	239	tributory of Black Rivulet	-41.19191	147.79211	420	C1
252	240	Black Rivulet	-41.17581	147.78256	283	C1
253	241	Evercreech Forest Reserve	-41.40284	147.97150	367	NE1
254	242	Weavers Creek	-41.42698	147.36859	577	C1

255	243	Weavers Creek	-41.42960	147.36905	554	C1
256	244	Weavers Creek	-41.42493	147.36874	577	C1
257	245	Weavers Creek Road	-41.39314	147.36293	905	C1
258	246	Weavers Creek Road	-41.39851	147.37648	1015	C1
259	247	tributory of Weavers Creek	-41.40383	147.38327	995	C1
260	248	tributory of Weavers Creek	-41.40990	147.37686	904	C1
261	249	Mount Barrow Road	-41.35911	147.40802	737	C1
262	250	Mount Barrow Road	-41.35573	147.42249	780	C1
263	251	Mount Barrow Road	-41.37246	147.42865	929	C1
264	252	Mt Barrow	-41.37832	147.42387	1262	C1
265	253	St Patricks River near Targa	-41.31208	147.36844	406	NE1
266	254	St Patricks River	-41.28357	147.38280	424	NE1
267	255	The Sideling	-41.27068	147.37875	508	NE1
268	256	The Sideling	-41.26799	147.39401	589	NE1
269	257	Headleys Creek, The Sideling	-41.27266	147.41893	589	NE1
270	258	Myrtle Grove Forest Reserve	-41.26896	147.50557	311	NE1
271	259	Myrtle Grove Plantation	-41.29133	147.49107	578	NE1
272	260	Myrtle Grove Forest Reserve	-41.27025	147.49579	283	NE1
273	261	St Patricks River	-41.29895	147.38217	415	C1
274	262	Mount Scott	-41.31279	147.50906	753	NE1
275	263	Mount Scott	-41.29787	147.50575	726	NE1
276	264	East Diddleum Road	-41.30044	147.46973	605	NE1
277	265	Diddleum Road	-41.32588	147.46909	597	C1
278	266	Diddleum Road	-41.33511	147.49229	634	C1
279	267	Mt Maurice	-41.30872	147.58835	1117	NE1
280	268	Mt Maurice	-41.30715	147.58466	1100	NE1
281	269	Mt Maurice	-41.31173	147.56259	922	NE1
282	270	Knights Road	-41.31487	147.53867	803	NE1
283	271	Upper St Patricks River	-41.33314	147.53534	636	NE1
284	272	Wayback Hill	-41.33311	147.58981	941	NE1
285	273	Maurice Road	-41.32775	147.61180	907	NE1
286	274	Maurice Road	-41.32458	147.62997	685	NE1
287	275	Russels Road	-41.35015	147.58545	728	NE1
288	276	Simons Road	-41.35338	147.51909	788	NE1
289	277	Evercreech Forest Reserve	-41.40494	147.98264	379	NE1
290	278	Mathinna Falls	-41.40593	147.89558	393	NE1
291	279	Mathinna Falls	-41.40577	147.89561	399	NE1
292	280	Dilgers Hill Rd	-41.37545	147.91234	667	NE1
293	281	Mt Albert Rd, near Mt Young	-41.37105	147.92178	677	NE1
294	282	Mt Albert Rd-near Mt Young	-41.35273	147.98830	721	NE1
295	283	Back Gully Rd	-41.37577	147.97903	641	NE1
296	284	Back Gully Rd	-41.35820	147.98457	709	NE1
297	285	Mt Albert Rd-near Mt Young	-41.35068	147.97635	725	NE1
298	286	Mt Albert Rd, near Mt Young	-41.34925	147.96727	728	NE1
299	287	Mt Young	-41.35707	147.95056	754	NE1
300	288	Mt Albert Rd	-41.36974	147.88638	682	NE1
301	289	Caves Creek, near Mt Victoria	-41.34449	147.86095	794	NE1
302	290	Mt Albert Rd, Mt Victoria	-41.34622	147.84312	843	NE1

303	291	Mt Albert Rd, near Mt Victoria	-41.34693	147.82970	807	NE1
304	292	Mt Victoria	-41.33465	147.83383	1045	NE1
305	293	Mt Victoria	-41.33714	147.82863	969	NE1
306	294	Mt Victoria	-41.34147	147.82278	856	NE1
307	295	Una Plain	-41.34421	147.80736	815	NE1
308	296	Mt Albert Rd	-41.34579	147.79420	811	NE1
309	297	Mathinna Plains Road	-41.31714	147.75595	805	NE1
310	298	Mathinna Plains Road	-41.31308	147.75168	766	NE1
311	299	Ben Ridge Road	-41.34541	147.73792	847	NE1
312	300	Ben Ridge Road	-41.34980	147.72786	861	NE1
313	301	Paradise Plains	-41.35394	147.71802	871	NE1
314	302	Ding Dong Hill	-41.35802	147.69876	844	NE1
315	303	Ben Ridge Rd	-41.34819	147.67050	879	NE1
316	304	Ben Ridge Rd	-41.34860	147.65356	864	NE1
317	305	Two Shed Plain	-41.35226	147.63831	918	NE1
318	306	Mathinna Plains Road	-41.37118	147.77527	791	NE1
319	307	Nile River	-41.59699	147.55145	567	C1
320	308	Myrtle Vale	-41.58269	147.61191	834	C1
321	309	Rabbit Marsh Creek	-41.59160	147.59335	821	NE1
322	310	Mt Albert summit	-41.34908	147.87239	1050	NE1
323	311	Mt Albert summit	-41.34882	147.87151	1040	NE1
324	312	McGoughs Lookout, Blue Tier	-41.15974	148.00388	717	C1
325	313	McGoughs Lookout, Blue Tier	-41.15518	148.00554	725	C1
326	229	Sun Flats Road	-41.19770	148.03081	670	C1
327	229	Sun Flats Road	-41.19769	148.03058	670	NE1
328	229	Sun Flats Road	-41.19757	148.03026	671	C1
329	314	Sun Flats Road	-41.19689	148.02982	678	C1
330	315	Frome Road	-41.15990	147.92604	516	C1
331	316	Frome Road	-41.16550	147.93698	517	C1
332	317	Frome Road	-41.16987	147.94002	519	C1
333	318	South George River	-41.31079	147.94240	236	NE1
334	319	St Columbia Falls	-41.32166	147.92270	289	NE1
335	320	Forest Lodge Rd	-41.28833	147.91374	564	NE1
336	321	Dawsons Creek	-41.23533	147.95340	549	NE1
337	322	Maa Louy Rd	-41.23087	147.88745	708	NE1
338	323	Rattler ranges	-41.24243	147.84546	809	NE1
339	324	Rattler ranges	-41.23719	147.83687	821	NE1
340	325	Ben Nevis	-41.39993	147.63188	1182	NE1
341	326	Mount Arthur	-41.27654	147.28902	1100	NE1
342	327	Mount Arthur	-41.27587	147.28036	1051	NE1

Appendix 3

Amplification conditions and the approximate size (bp) of the PCR product for each primer pair that successfully amplified *N. cunninghamii* cpDNA.

Fragment	Annealing Temperature (˚C)	Approximate size (bp) in *N. cunninghamii*	Author
trnD-trnT	54	1000	a
trnS-trnf$_M$	64	1200	a
trnK-trnQ	57	1400	b
rpoC1-trnC	50	3200	b
trnV-rbcL	57	3000	b
rpl23-psbA3	48	3200	c
atpH-atpI	50	1200	c
atpI-rpoC2	60	2000	c
rpoC2-f-*rpoC2*-r	58	2700	c
orf184-petA	52	2600	c
petA f-*psbE* r	42	1900	c
clpp-psbB	51	2500	c
psbB-petB	52	2500	c
petB-petD	55	1800	c
trnH-trnK	62	1800	a
trnK-trnK	58	2300	a

a) Demesure *et al.* (1995); b) Dumolin-Lapegue *et al.* (1997); c) Grivet *et al.* (2001)

Chapter 3: Strong phylogeographic structure of the bird-dispersed shrub *Tasmannia lanceolata* (Winteraceae)

Introduction

It has long been recognised that characteristics of plant propagules (seed, fruit, spores and vegetative disseminules) are likely to affect the ability of plants to disperse long-distances (Darwin, 1859; Ridley, 1930; Carlquist, 1967; Sorensen, 1986; Jordan, 2001). In general, species lacking specialised features for dispersal (gravity dispersed propagules) have been viewed as having the least dispersal potential. On the other hand, plants with modifications for dispersal by wind and mobile animals, particularly birds, are often considered to have high potential for longer distance movements (Ellner & Shmida, 1981; Horn *et al.*, 2001; Howarth *et al.*, 2003). However, uncertainty remains as to how these traits relate to effective dispersal (i.e. dispersal leading to viable and continuing populations) and, therefore, the importance of these traits in determining the relative mobility of plants including their capability of migration.

A classic example of this problem are plants that bear fleshy fruit, a propagule type considered to be adapted for dispersal by vertebrates, especially birds (Snow, 1970; Van Der Pijl, 1982). This trait is common in the tropical and temperate floras of the world (Willson *et al.*, 1990) and is thought to provide an advantage enabling the targeted dispersal of seed by birds into habitat favourable for establishment (McKey, 1975; Howe & Smallwood, 1982; Wenny & Levey, 1998; Bolmgren & Eriksson, 2005) and high effective dispersal into both disturbed and undisturbed environments (Gade, 1976; Vitousek & Walker, 1989; Rejmánek, 1996; Atkinson & Atkinson, 2002; Nishi & Tsuyuzaki, 2004). Thus bird-dispersal is thought to allow the maintenance of large metapopulations across species ranges (Lord, 1999). However, recent evidence suggests that effective long-distance dispersal of fleshy fruited propagules is no more common than comparable dispersal of those that are gravity dispersed (Jordan, 2001; Higgins *et al.*, 2003). Fleshy fruited plants were found to be significantly under-represented compared to species with other or no obvious dispersal traits in the herbaceous flora shared between the island of Tasmania, Australia, and New Zealand

(Jordan, 2001). Similar observations have been observed in the flora of the oceanic island of Juan Fernandez in comparison to its major source area, the Chilean mainland (Bernadello *et al.*, 2006).

In recent decades the long-distance dispersal capabilities of plants with different propagule traits have been investigated by examining the spatial patterns and phylogenetic relatedness of genetic variation (or phylogeographic structure) across the ranges of species. Of particular importance have been studies of the usually seed dispersed chloroplast DNA (Mogensen, 1996). Long-distance dispersal is considered crucial to determining genetic structure of plants (Cain *et al.*, 2000), and the spatial structuring of chloroplast haplotypes across species ranges can contain a strong record of past effective seed dispersal (McCauley, 1995). Gravity dispersed plants have generally displayed strong phylogeographic structuring (Duminil *et al.*, 2007) whereas wind dispersed plants have usually been characterised by low phylogeographic structuring of chloroplast haplotypes, for example, *Salix caprea* (Palme *et al.*, 2003b), *Betula pendula* and *B. pubescens* (Palme *et al.*, 2003a; Maliouchenko *et al.*, 2007) and *Populus tremula* (Salvini *et al.*, 2001).

However, the effective dispersal ability and genetic consequences of fleshy fruitedness are less certain. Multiple chloroplast phylogeographic studies have observed low levels of spatial structuring in chloroplast DNA of fleshy fruited species in Europe (Raspe *et al.*, 2000; Oddou-Muratorio *et al.*, 2001; Mohanty *et al.*, 2001 2002; Fineschi *et al.*, 2005b). These results have been viewed as being consistent with expectations of the bird-dispersal of these fleshy fruited plants (e.g. Fineschi *et al.*, 2005b), that is, having enabled frequent effective long-distance dispersal of seed across the distribution of these species. However, contrasting results have been observed for other fleshy fruited plants in Europe. Grivet & Petit (2002) observed significant phylogeographic structure within ivy (*Hedera* sp.) and considered that this may be due to the resistance to invasion of established populations as a result of the presence of large clones created by vegetative reproduction. In addition, extensive admixture of chloroplast lineages has not occurred during the postglacial dispersal of holly (*Ilex aquifolium*; Rendell & Ennos, 2003) and *Rosa pendula* (Fer *et al.*, 2007). In a study of the fleshy-fruited European shrub alder buckthorn (*Frangula alnus*), despite chloroplast evidence for high seed mobility and admixture in the extensive central and northern European populations of this species, strong phylogeographic

structuring was found in the southern extremes of the species range (Hampe *et al.*, 2003). This was explained by the absence of migratory birds in these southern populations (Hampe *et al.*, 2003). Outside of Europe the phylogeographic structure of bird-dispersed plants have been poorly studied. However, (Rossetto *et al.*, 2009) identified evidence for strong limitations of dispersal at nuclear microsatellite loci across the range of multiple small fruited species within the genus *Elaeocarpus* in the rainforest of northeast Queensland (Australia) which are likely to have limited pollen movement (Rossetto *et al.*, 2004).

The aim of this study is to contribute to our understanding of the consequences of possessing fleshy fruit for dispersal by birds and their effect on effective plant dispersal by investigating the chloroplast phylogeography of *Tasmannia lanceolata* (Poir.) A.C. Smith (Winteraceae), a widespread and patchily distributed shrub of mesic habitats in southeastern Australia. As in other members of the Winteraceae (Gottsberger *et al.*, 1980; Norton, 1982; Lloyd & Wells, 1992; Armesto *et al.*, 2001), *T. lanceolata* possesses small berry like fruit (Fig. 1) that are consumed by birds (Read & Hill, 1983; Read, 1989; Cash, 1998; Borzak, 2003). The fruit are 3.0–7.0 mm in diameter and contain 2-18 shiny and irregular shaped seed (Floyd, 1989; Raleigh *et al.*, 1994).

Fig. 1 Berry-like fruit of *Tasmannia lanceolata* (Poir.) A.C. Smith (Winteraceae).

Tasmannia lanceolata co-occurs with, and has ecological similarities to *Nothofagus cunninghamii*, the dominant cool temperate rainforest tree in south-eastern Australia. However, *N. cunninghamii* has gravity dispersed seed (Howard, 1973; Hickey *et al.*, 1982), and the strong chloroplast phylogeographic patterns observed across the distribution of this species has provided evidence that *N. cunninghamii* withstood glacial climates within multiple regions with minor population expansion out of glacial refugia during the Holocene (Chapter 2). Thus, if bird-dispersal has facilitated effective long-distance dispersal, one would expect *T. lanceolata* to have considerably less strongly structured phylogeography than *N. cunninghamii*. However, (Barnes *et al.*, 2000) showed strong spatial patterning in cuticular morphology within *T. lanceolata* (Fig. 2), and argued that because the boundary between cuticular morphotypes transgressed climatic and edaphic gradients the species retains historical patterning, which would imply limits to effective seed dispersal. However, selection could not be completely discounted as a possible cause of this spatial pattern.

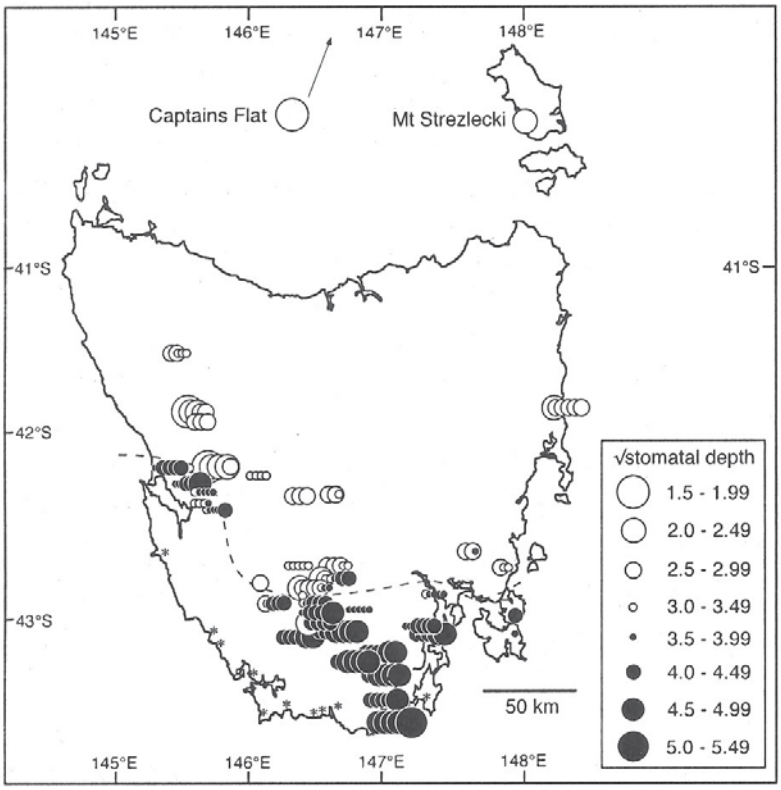


Fig. 2 The distribution of stomatal forms observed in *Tasmannia lanceolata*. The size of each circle indicates the depth of encryption for a single plant. Asterisks represent herbarium records of plants all with the southern cuticle morphology. The dashed line indicates the approximate location of the boundary between southern (encrypted) and northern (superficial) forms. A single population sampled at Captains Flat is located in southern New South Wales. From Barnes *et al.* (2000).

This study aimed to test the hypothesis that fleshy fruitedness has allowed *Tasmannia lanceolata* to have undergone extensive movement within and between parts of the species range including across areas of unsuitable habitat. If this is true we would expect (a) low phylogeographic structure with chloroplast lineages not associated with particular landscapes, (b) significant levels of admixture of different chloroplast haplotypes at multiple spatial scales, and (c) that disjunct populations would harbour haplotype(s) that are most frequent in nearby populations.

Materials and Methods

Species description

Tasmannia lanceolata (mountain pepper) is a dioecious (Curtis & Morris, 1993), insect pollinated (Thien, 1980) (P. McQuillan pers. comm.) shrub or small tree occasionally reaching heights of 10 m (Floyd, 1989). It is distributed across ~ 10 degrees of latitude within the mountainous regions of southeastern Australia and the island of Tasmania (Fig. 3) where annual rainfall exceeds ~ 930 mm (Sniderman, 2007). The species is considered a common early successional species of disturbed and open habitats (Read & Hill, 1983; Read, 1989) and a colonizer of canopy 'gaps' in closed forests (Ellis, 1985). The maximum stem lifespan of the species is unknown it is unlikely to exceed 200 years. Also, *T. lanceolata* is capable of vegetative growth from basal sprouts, as has been observed in two other *Tasmannia* species (Campbell & Clarke, 2006). Within its distribution *T. lanceolata* occurs in a wide range of vegetation types including creek lines of dry *Eucalyptus* forest, wet *Eucalyptus* forest understorey, near the margins or scattered within the understorey of cool temperate rainforests, and in Tasmania, alpine shrublands (Vink, 1970). Apart from differences in stomatal encryption observed by (Barnes *et al.*, 2000) and clines in leaf size and shape along altitudinal gradients in Tasmania, *T. lanceolata* is markedly uniform across its range (Casey, 1991; Barnes, 1995).

Sampling strategy

Leaves were sampled from 244 *T. lanceolata* individuals from across almost the entire distribution of the species (Fig. 2). A total of 230 of these individuals were sampled more than 400 m apart. However, in the most isolated and smallest populations more than one individual was taken where possible, with a minimum of 10 m between sampled individuals. At five locations in Tasmania (Wylds Craig, Mt

Barrow, Snowy South, Mt Wedge and Mt Arrowsmith) both forest understorey and alpine forms of *T. lanceolata* were collected. For a list of all samples collected see Appendix 1. Information on the distribution of *T. lanceolata* was obtained from a number of sources including the Natural Values Atlas (Department of Primary Industries and Water, Tasmanian Government (http://www.naturalvaluesatlas.dpiw.tas.gov.au) Australia's Virtual Herbarium (http://www.anbg.gov.au/avh/cgi-bin/avh.cgi), Floyd (1989, 1990) and personal knowledge.

There are 8 *Tasmannia* species in Australia and ~ 50 species within the genus (Wilson, 2007). This project sampled all seven Australian species that occur in the southeastern part of the continent in order to examine the potential for chloroplast exchange between *T. lanceolata* and other species. While *T. lanceolata* is morphologically distinct from other Australian species (Sampson *et al.*, 1988; Raleigh *et al.*, 1994) hybridisation has been reported between *Tasmannia* species in Australia and Papua New Guinea (Vink, 1970; Sampson *et al.*, 1988) and among species of other Winteraceae genera (Ehrendorfer F, 1979; Sampson, 1980). Historical hybridisation can lead to sharing of haplotypes among species (Dixon *et al.*, 2007), complicating the interpretation of phylogeographic patterns (Alvarez & Wendel, 2006). Therefore, in order to examine whether chloroplast exchange has contributed to the chloroplast patterns of *T. lanceolata,* multiple samples were taken from all species that have overlapping distributions with *T. lanceolata*. These were *T. xerophila* subsp. *xerophila* (5 samples from 5 localities), the east Gippsland endemic *T. xerophila* subsp. *robusta* (3 samples from one locality) and the Victorian central highlands endemic *T. vickeriana* (2 samples from one locality) (Fig. 2). In addition, one sample each of other Australian species *T. glaucifolia, T. purpurascens* and *T. stipitata* and three samples of *T. insipida* were included. These taxa are hereafter referred to as the 'other *Tasmannia* taxa'. For use as outgroups, one sample each of the New Zealand endemic *Pseudowintera colorata* (cultivated at the University of Tasmania) and the Chilean endemic *Drimys winteri* (grown in a private collection by Ken Gillanders, southern Tasmania) were used. Another outgroup was provided by the complete sequence of the cpDNA genome of *D. granadensis* in GenBank (GenBank accession number DQ887676; Cai *et al.*, 2006).

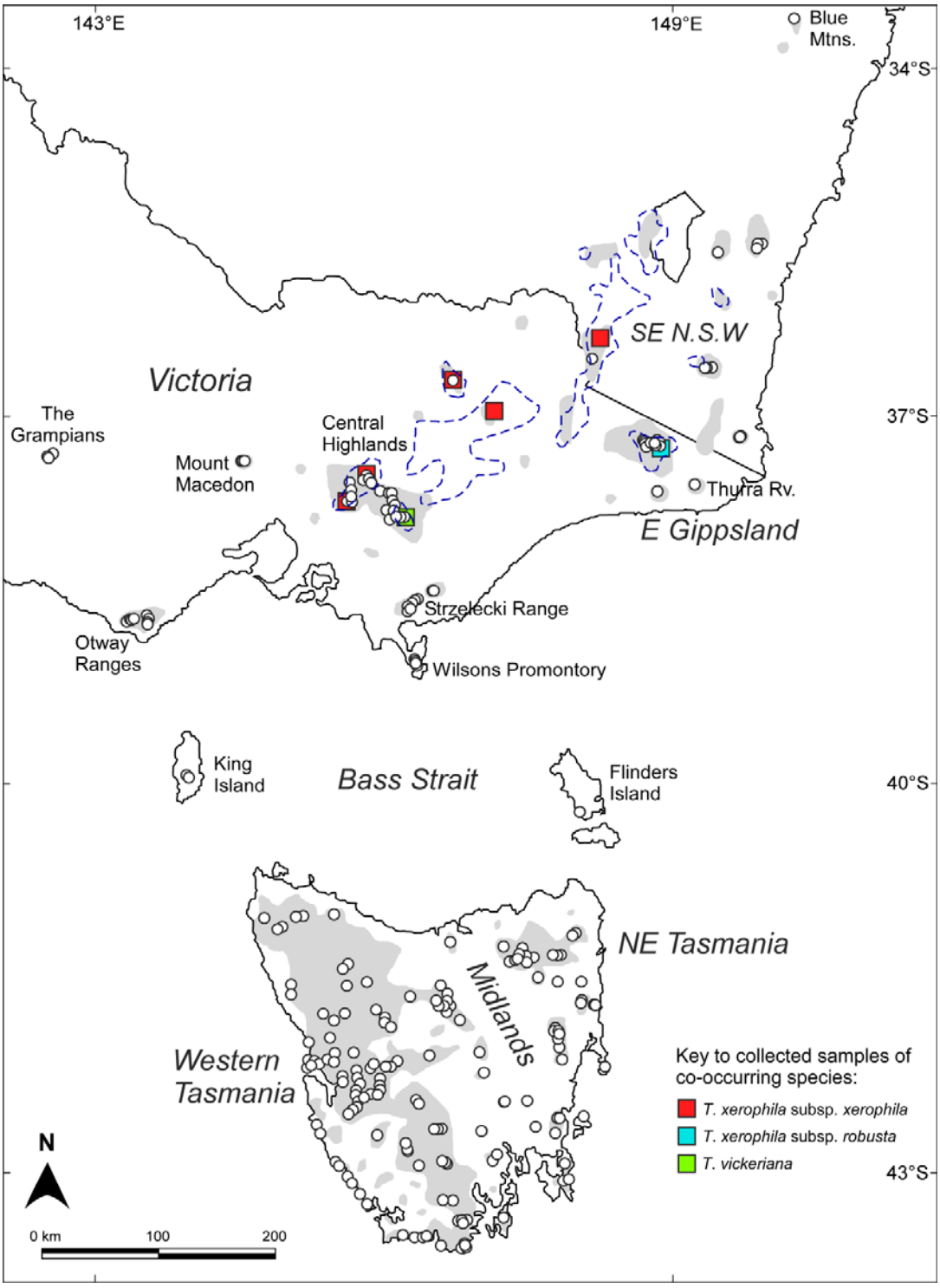


Fig. 3 Natural distribution of *Tasmannia lanceolata* (grey shading). Each white circle indicates the location of a sample of *T. lanceolata* (in some cases 2-3 samples) included in this study. The blue broken line encloses the distribution of three *Tasmannia* taxa that have overlapping distributions with *T. lanceolata*. Collections of these taxa are indicated by square symbols.

Molecular methods

Total genomic DNA was extracted from 0.25 g of adult leaves using the Qiagen DNeasy Plant Mini Kit (QIAGEN Pty Ltd Vic, Australia). DNA quantity and quality were assessed by agarose gel electrophoresis with ethidium bromide staining and comparison with a standard molecular weight marker (Lambda *Hind*III).

For all 244 *T. lanceolata* samples, samples of other *Tasmannia* taxa, and outgroup samples, six regions of chloroplast DNA were amplified using PCR. These regions were: the *pet*N–*psb*M and *psb*M–*trn*D intergenic spacers using the primer pairs *pet*N1-*psb*M2R and *psb*M2-*trn*D (Lee and Wen 2004); the *trn*L intron and the *trn*L–*trn*F intergenic spacer using the primer pairs c-d and e-f (Taberlet 1991); and the *trn*K intron (including part of the *mat*K gene) using the primers K1-*mat*K1 and *mat*K6-K2 (Demesure *et al.*, 1995; Grivet & Petit, 2002). All PCR reactions were performed in a total volume of 25 μl containing 2.5 mM $MgCl_2$; 100 μg/mL of Bovine Serum Albumin; 80 μM each of dATP, dCTP, dGTP and dTTP; 5 pM of each primer; 1 x PCR buffer (67 mM Tris-HCl, 16.6 mM $(NH_4)_2 SO_4$, 0.5% Triton X-100 and 5 μg of gelatin); two units of *Taq* DNA polymerase; and approximately 10 ng of genomic DNA (1-2 μL of gDNA). PCR amplifications were performed using a MJ Research PTC-225 Thermal Cycler (GMI, Inc. Minn., USA). PCR conditions were as follows: for *psb*M2-*trn*D and *pet*N1-*psb*M2R reactions, an initial 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 2 mins at 51°C and extension for 2 min at 72°C, and a final extension step for 10 min at 72°C; for e-f and c-d, an initial 1 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 50°C and extension for 45 secs at 72°C, and a final extension step for 7min at 72°C; for K1-*mat*K1 and *mat*K6-K2, an initial 4 min at 94°C, followed by 35 cycles of 45 sec at 94°C, 1 min at 51°C and extension for 1.5 min at 72°C, and a final extension step for 10 min at 72°C.

PCR products were purified using the Qia-Quick PCR purification kit (QIAGEN Pty Ltd Vic, Australia). DNA sequencing was performed in one direction using the forward primers *pet*N1, c, K1 and *mat*K6 and the reverse primers *trn*D and f. A large deletion of 447 bp was found that distinguished *T. lanceolata psb*M–*trn*D sequences from those of all other *Tasmannia* and outgroup taxa. To obtain longer read length with these other taxa an internal forward primer (5' ACTCGGGATCTAATCCCATAGAGA 3') was used.

Sequencing reactions were performed in a MJ Research PTC-225 Thermal Cycler using ABI Prism Bigdye Terminator v 3.0 Cycle Sequencing Kits (Applied Biosystems, CA, USA) with AmpliTaq DNA polymerase (Applied Biosystems, CA, USA) and run on a 3730*xl* DNA Analyzer (Applied Biosystems, CA, USA). Sequences were aligned using Sequencher 4.5 (Gene Codes Corporation, MI, USA), and checked by eye for incorrect base calls, base pair (bp) substitutions and DNA insertions or deletions (indels). DNA polymorphisms detected in only one sample (including those found in the outgroup taxa and the single samples of *T*. *stipitata* and *T*. *purpurascens* (except for *T*. *glaucifolia*) were validated by repeating both the PCR and the sequencing reaction. Gaps were positioned to maximise conformity to known indel types such as simple and inverted repeats of adjacent sequences (Crayn *et al.*, 2006) and to minimise the number of inferred indel events (Graham *et al.*, 2000). Two overlapping indels were scored as multistate characters. Characters that occurred only in the other *Tasmannia* taxa and outgroup samples within the 447 bp section of sequence that was not present in the *psb*M–*trn*D intergenic spacer sequences of *T. lanceolata* were not included in the data set.

Test for neutrality and spatial structuring

Neutrality was tested for each of the six fragments separately using Tajima's *D* (Tajima, 1989) and Fu and Li's *D** and *F** statistics (Fu & Li, 1993) undertaken using DnaSP 4.90.1. (Rozas *et al.*, 2003) using all 244 *T. lanceolata* samples. The spatial structuring of *T. lanceolata* haplotypes, clades and subclades was investigated using the single nearest geographic neighbour for each sample was determined using a specially written macro in SAS 9.1 (SAS Institute Inc., Cary, NC, USA). This program also performed a permutation test (Manly, 1997) with 10,000 randomised repeats testing whether the nearest neighbour of each sample was more often of the same haplotype, subclade and clade than expected by chance (a proxy of spatial structure). In order to do this analysis 14 samples that occurred less than 400 metres apart (mostly affecting representative samples from isolated populations) were excluded. Samples that occurred less than 400 m apart were deleted randomly from the dataset except in three cases where samples possessed different haplotypes were not excluded from the nearest neighbour analysis.

Phylogenetic estimation of cpDNA haplotypes

Maximum parsimony (MP) analysis of cpDNA haplotypes were undertaken using the program PAUP* version 4.0b10 (Swofford, 2000). All characters, including indels, were treated as unordered and of equal weight. The MP analysis used a heuristic search with 1000 replicates of stepwise, random branch swapping addition sequence followed by tree-bisection-reconnection (TBR). Branch support was assessed by bootstrap analysis (Felsenstein, 1985) with 100 bootstrap replicates using the same search parameters as those in the parsimony analysis except that 10 heuristic search replicates were undertaken instead of 1000. In addition, 'max trees' was set to 1000 due to computer memory overload. *Pseudowintera colorata*, *D. granadensis* and *D. winteri* were used as outgroups.

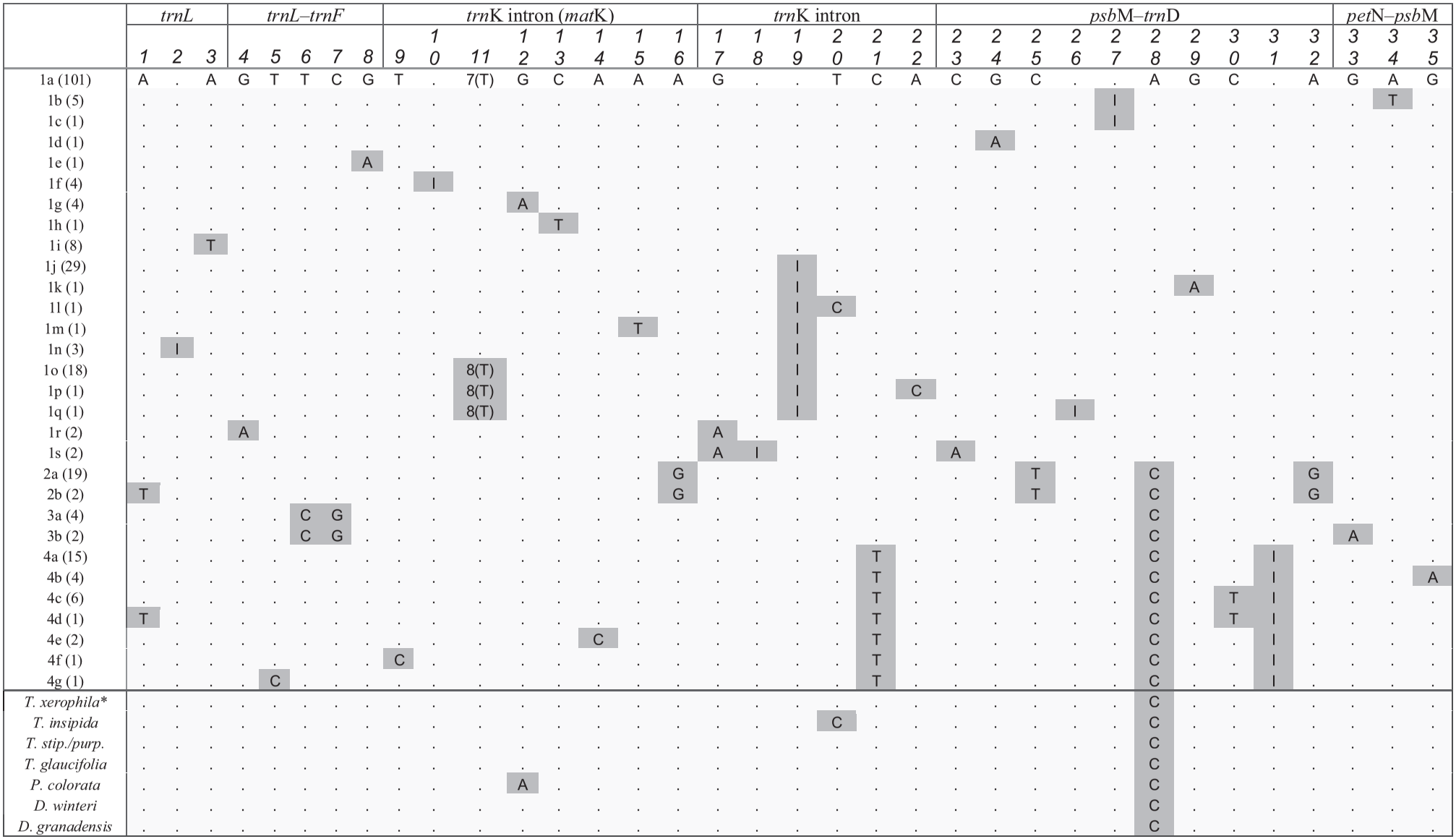
A three bp length inversion with perfect flanking inverted repeats (IR) of 14 bp and ΔG = -14.3 kcal/mol (calculated using OligoAnalyzer 3.1 http://test.idtdna.com/analyzer/Applications/OligoAnalyzer/) was discovered within the *psb*M–*trn*D intergenic spacer. Such short inversions associated with long IR's have high hairpin stability (Kelchner & Wendel, 1996; Catalano *et al.*, 2009) and are often highly homoplastic (Kelchner & Wendel, 1996; Sang *et al.*, 1997; Graham *et al.*, 2000; Quandt *et al.*, 2003). The inversion identified in this study was inferred as having shifted states at least four times within *T. lanceolata* and four times within other taxa (data not shown). It was excluded from all analyses. In addition, for all *T. lanceolata* haplotypes a median-joining network, with equal weighting of all characters, was constructed using *Network 4.5.0.2* (Bandelt HJ *et al.*, 1999).

Results

Chloroplast variation

A total of 3,190 bp of aligned sequence (equal to 1.98 % of the *Drymis granadensis* chloroplast genome (Cai *et al.*, 2006)) was sampled. Overall 157 single base pair nucleotide or indel polymorphisms were observed in the whole data set, of which 35 occurred among samples of *T. lanceolata* defining 30 chloroplast haplotypes (Table 1). *Tasmannia lanceolata* polymorphisms consisted of 27 single nucleotide polymorphisms (23 transitions and 4 transversions) and 8 indels varying in length from 1 bp to 8 bp of which five were exact repeats of the preceding DNA sequence.

Table 1 Single base pair nucleotide polymorphisms and insertions/ deletions characterising all 30 chloroplast DNA haplotypes observed in *Tasmannia lanceolata*, shown in comparison to the most frequent haplotype 1a. The states at variable sites in *T*. *lanceolata* are also shown for other *Tasmannia* species and *Drimys* and *Pseudowintera.* **T. xerophila* includes the subspecies *T. xerophila* subsp. *xerophila* and *T. xerophila* subsp. *robusta* and the closely related *T. vickeriana*.


trnL
trnL–trnF
trnK intron (matK)
trnK intron
psbM–trnD
petN–psbM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
1a (101) A . A G T T C G T . 7(T) G C A A A G . . T C A C G C . . A G C . A G A G
1b (5)
1c (1)
1d (1)
1e (1)
1f (4)
1g (4)
1h (1)
1i (8)
1j (29)
1k (1)
1l (1)
1m (1)
1n (3)
1o (18)
1p (1)
1q (1)
1r (2)
1s (2)
2a (19)
2b (2)
3a (4)
3b (2)
4a (15)
4b (4)
4c (6)
4d (1)
4e (2)
4f (1)
4g (1)
T. xerophila*
T. insipida
T. stip./purp.
T. glaucifolia
P. colorata
D. winteri
D. granadensis

Partial aligned sequence lengths obtained for *T. lanceolata* were as follows: the *trn*L intron 491- 496 bp; the *trn*L–*trn*F intergenic spacer 368 bp; the *pet*N1-*psb*M intergenic spacer 331 bp; the *psb*M–*trn*D intergenic spacer 677 - 683 bp; and for the *trn*K intron , K1–*mat*K1 598 - 606 bp and *mat*K6-K2 700 - 706 bp. The Tajima's *D* and Fu and Li's *D** and *F** statistics neutrality test were all insignificant, indicating there is no reason to believe that the cpDNA of *T. lanceolata* is under selection. All variant sequences within *T. lanceolata* and other *Tasmannia* taxa and outgroups were deposited in Genbank (see Appendix 2).

Phylogenetic relationships of Tasmannia

Parsimony analysis of the full cpDNA dataset yielded 151 most parsimonious trees. All *T. lanceolata* haplotypes and other *Tasmannia* taxa formed a strongly supported clade (bootstrap percentage, BP=100%; Fig**.** 4) defined by 53 polymorphisms from *P. colorata*, *D. winteri* and *D. granadensis* (Fig. 5).

The *Tasmannia* clade was split into two strongly supported clades (Fig. 4), one consisting of all 30 *T. lanceolata* haplotypes (Bootstrap percentage, BP=100%) and the other containing all other *Tasmannia* taxa (BP=99%). These two clades differed by a total of thirteen polymorphisms (8 base pair substitutions and 5 indels; Fig. 5). This suggests that hybridisation with other living species of *Tasmannia* has not played a significant role in the evolution of *T. lanceolata* and its phylogeography. The 30 haplotypes within *T. lanceolata* were divided into four clades with high to moderate bootstrap support (Fig. 4). The largest clade (clade 1) contained four haplotype subclades (subclades A, B, C and D) and clade 4 contained an additional subclade (subclade E). All subclades had moderate bootstrap support (Fig. 4).

In all cases where high and low altitude pairs were collected in western Tasmania they possessed different haplotypes but of the same clade, with the rarer haplotype at either low or high altitudes. However, the single high-low pair collected in northeast Tasmania shared haplotype 1a.

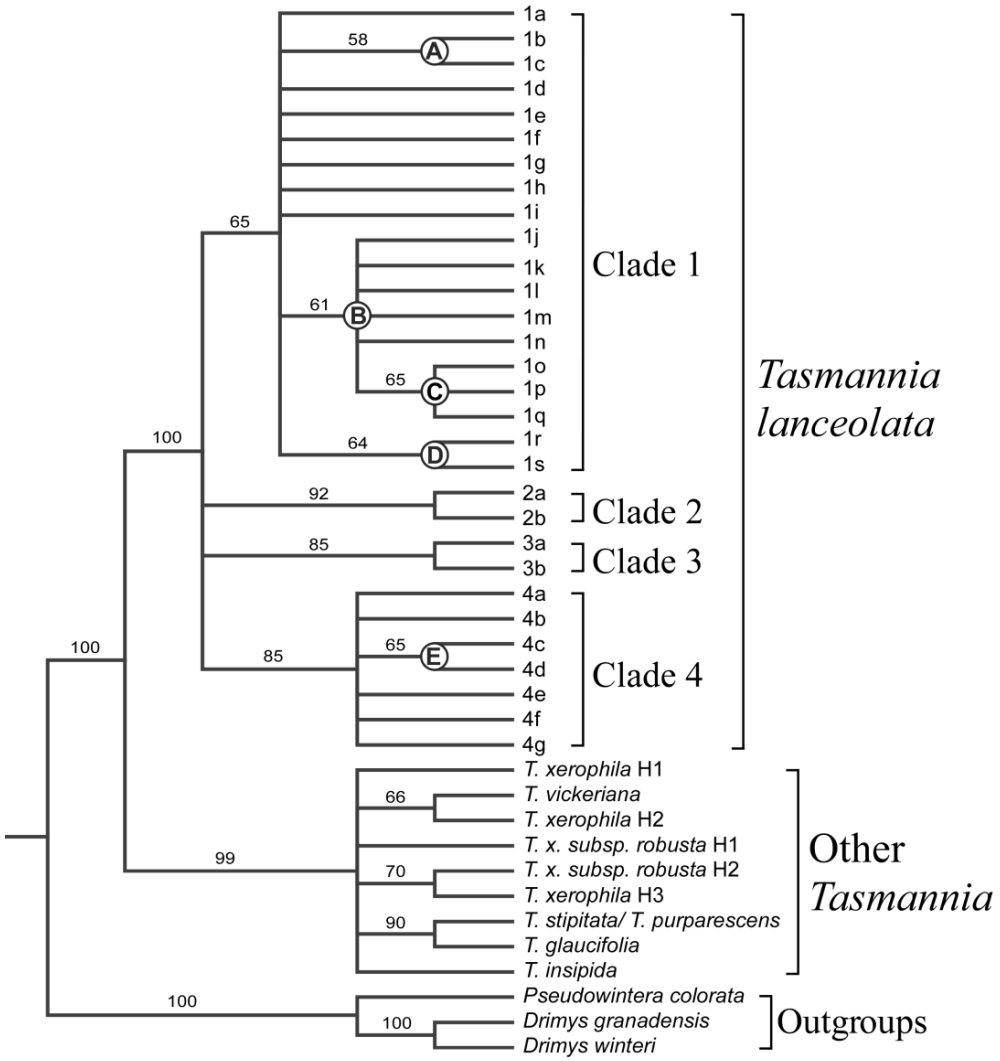


Fig. 4 The inferred phylogenetic relationships among *Tasmannia lanceolata* chloroplast haplotypes and their relationship to other Australian *Tasmannia* species and outgroups constructed via maximum parsimony analysis of 100 informative and 57 uninformative chloroplast characters. This tree is the strict consensus of 151 most parsimonious trees (L= 167, CI = 0.964, RI = 0.986, RC=0.963). Haplotype subclades within *T. lanceolata* are indicated with the letters (A, B, C, D and E). Bootstrap values above 50% are shown above branches. Haplotype names are the same as in Table 1.

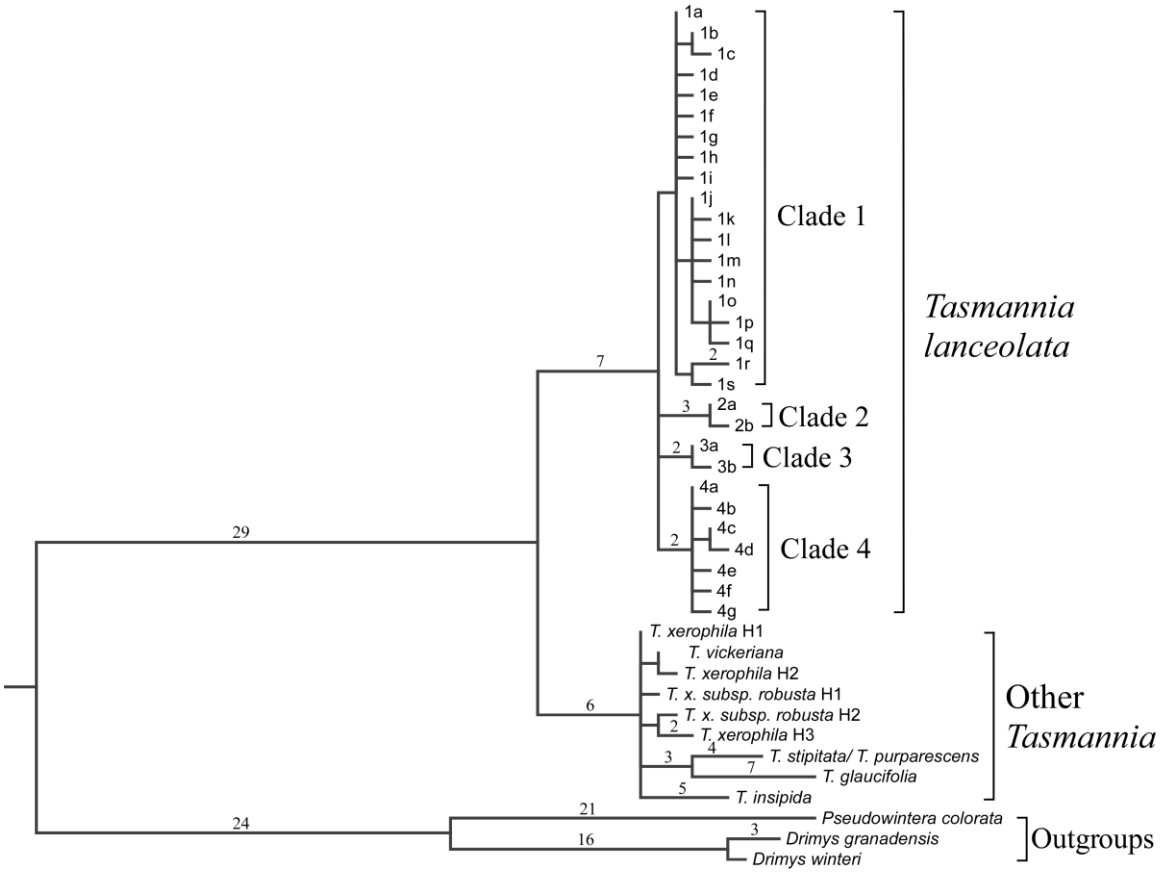


Fig. 5 One of the most parsimonious trees obtained from chloroplast sequence characters in *Tasmannia* and outgroups. Branch lengths (the inferred number of single base pair substitutions and indels on a branch) of greater than one are indicated above branches.

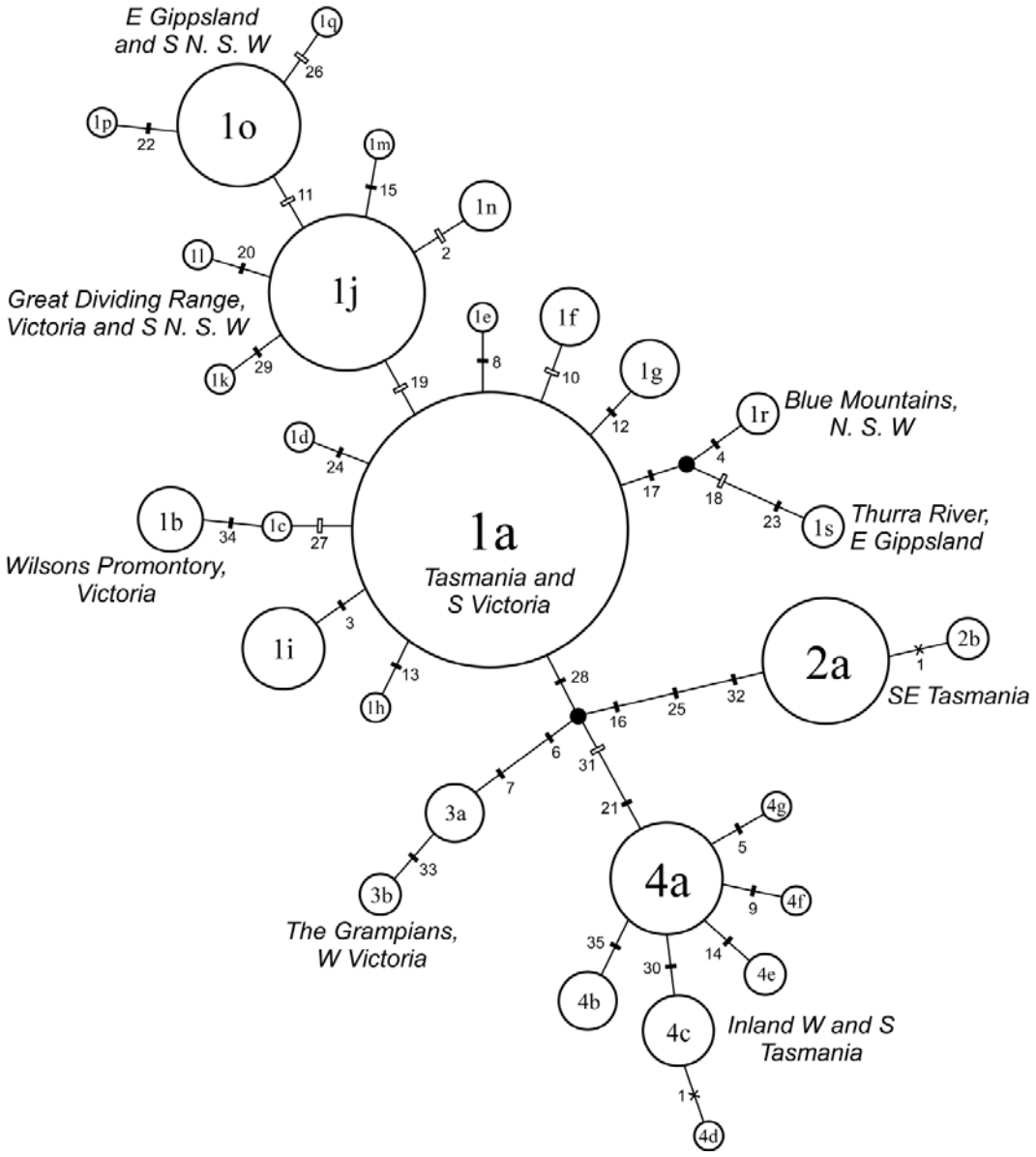


Fig. 6 Median-joining network of all *Tasmannia lanceolata* haplotypes. Each haplotype is represented by a circle and a number-letter combination as in Table 1. The size of a circle is proportional to the frequency of the haplotype as is indicated by the number below each name (except for singleton haplotypes). Lines connecting each haplotype are proportional to the number of character differences between them. Non-homoplasious single nucleotide base changes are indicated by solid bars, homoplasious single nucleotide base change by crosses, and indels (all non-homoplasious) by open bars. Small filled circles represent inferred haplotypes that were not sampled or are extinct.

Strong phylogeographic structuring of T. lanceolata *haplotypes*

The chloroplast variation observed within *T. lanceolata* displayed statistically significant phylogeographic structuring. The analyses of nearest geographic

neighbours showed that individuals were more likely ($P < 0.001$) to be geographically nearest to individuals of the same haplotype (156 of 230) or the same clade (210 of 230) than to individuals of different haplotypes or clades (Table 2). This level of patterning applied both within Tasmania and on the mainland.

Table 2 Nearest neighbour analysis of Tasmanian and mainland Australian samples of *Tasmannia lanceolata*. The number of observed individuals (*n*) with a nearest neighbour of the same clade (clades 1, 2, 3 or 4), subclade (A-E) and haplotype are shown with probabilities.

Nearest neighbour	Tasmania (n=155)			Mainland (n=75)		
	Observed	Expected	*P*	Observed	Expected	*P*
Of same clade	137 (88%)	79.2 (51%)	<0.001	73 (97%)	65.4 (87%)	<0.001
Of same subclade	130 (84%)	77.4 (50%)	<0.001	69 (92%)	22.9 (31%)	<0.001
Of same haplotype	104 (67%)	56.4 (36%)	<0.001	52 (69%)	16.0 (21%)	<0.001

This spatial structuring was further indicated by the fact that the species shows high haplotype diversity with virtually no overlap observed among the distributions of haplotypes. This lack of overlap applies not only to populations isolated by areas of unsuitable habitat but also to more or less continuous populations of the species within parts of Tasmania with no obvious barriers to seed flow. The only regions where overlap was observed were located (1) at the northern and southern edges of the range of clade 4 where haplotypes of both clade 1 and 4 were observed, and (2) in the Strzelecki Ranges of southern Victoria where haplotype 1a of clade 1 was in close contact with haplotypes of subclade B (Figs. 7 & 8).

Haplotypes of each clade and subclade within *T. lanceolata* were strongly associated with distinct geographical locations. Within each clade or subclade the most widespread haplotype was also observed to have the most connections to less frequent and derived haplotypes (Fig. 6). Three regions contain endemic clades: clade 4 (seven haplotypes and 12.3 % of samples) in inland western and southern Tasmania (Fig. 7); clade 2 (two haplotypes and 8.6% of samples) in the predominantly semi-humid southeast of Tasmania; and clade 3 (two haplotypes and

2.5% of samples) in the Mount William Range, the Grampians, Victoria (Fig. 7). The latter region is an isolated massif (~ 210 km from the nearest occurrence of the species at Mount Macedon and the Otway Ranges) with two very small populations of *T*. *lanceolata* (Fig. 3).

Haplotypes of clade 1 (76% of all samples) were extensively distributed in Tasmania and included all haplotypes of *T. lanceolata* observed on mainland Australia, excluding the Grampians populations. Within clade 1, haplotype 1a (41.4% of all *T. lanceolata* samples) was frequent, and often the sole haplotype, in the northeast, northwest and central west of Tasmania and also extending across the Bass Strait islands to the southernmost occurrence of *T. lanceolata* on mainland Australia (Otway Ranges, Wilsons Promontory and the Strzelecki Ranges) and along the western and southern Tasmanian coastline (Fig. 8). This haplotype was inferred to be ancestral within Clade 1 (note the zero branch length leading to it in Fig. 5). Strong phylogeographic structuring of haplotypes derived from haplotype 1a is evident with four subclades occurring in distinct geographical locations (Fig. 8) where haplotype 1a is rare or absent. Subclade A, consisting of two haplotypes (haplotypes 1b, 1c), was confined to the isolated occurrence of the species at Wilsons Promontory. Subclade B, consisting of haplotypes 1j to 1n (14.3% of all samples), occurred in the central highlands of Victoria to southern NSW while subclade C (which is nested within subclade B) was restricted to eastern Gippsland and parts of southern NSW (8.2%). A fourth subclade comprised two haplotypes, one in a population at Thurra River in eastern Gippsland (haplotype 1r) and the other in the disjunct and most northern part of the species range in the Blue Mountains, NSW (haplotype 1s). All other haplotypes observed more than once are also geographically restricted, for example, haplotype 1i in the Great Western Tiers of northern Tasmania and haplotype 1g in far southern Tasmania (Fig. 8). Some parts of the distribution of *T. lanceolata* contained no endemic haplotypes including the northeast, central west and coastal western Tasmania and the Bass Strait islands, and in southern NSW where only the most frequent haplotype of subclade B and C were observed.

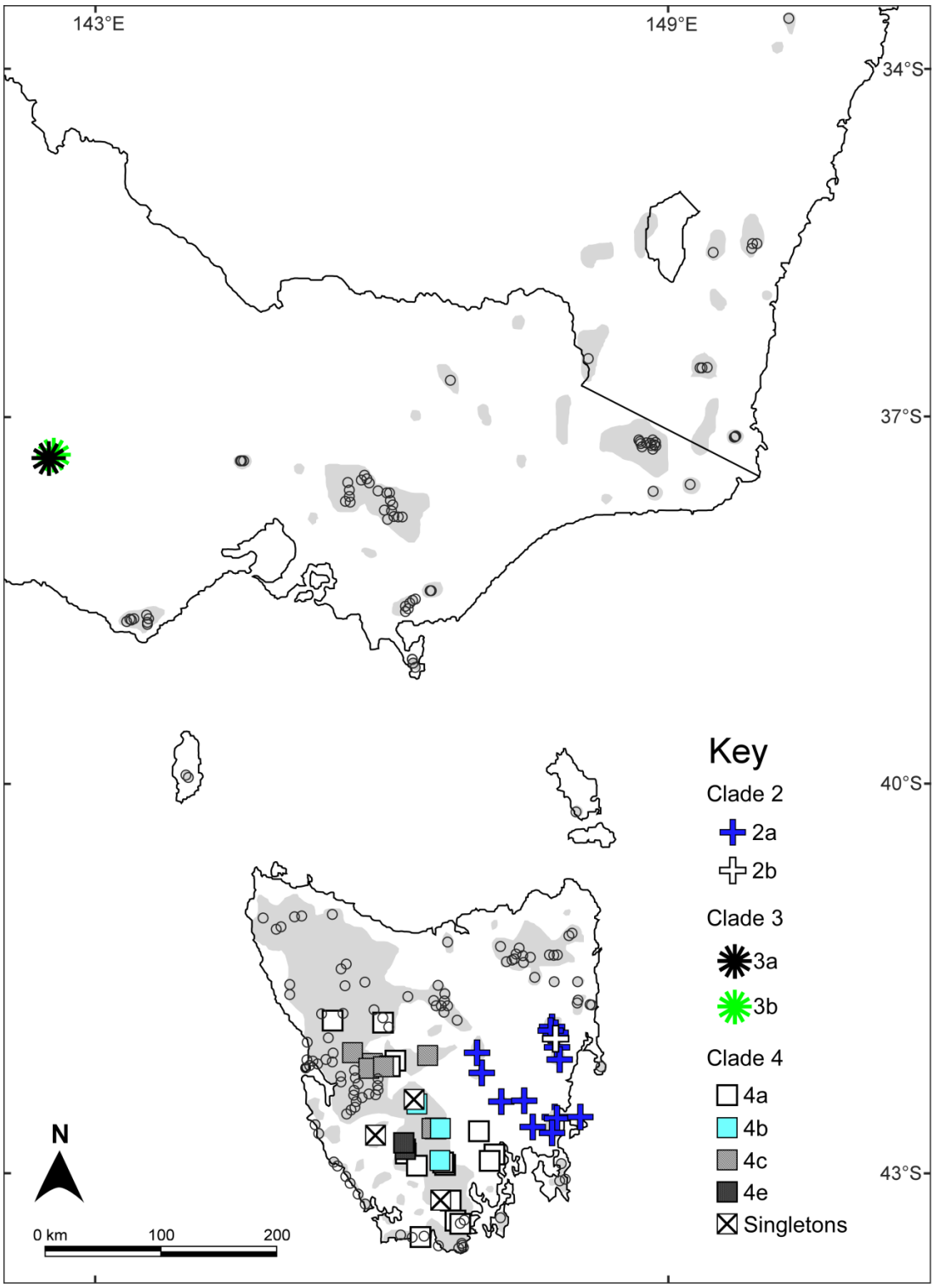


Fig. 7 Distribution of clades 2, 3 and 4 haplotypes observed in *Tasmannia lanceolata*. For reference, each sample location where haplotypes of clade 1 were observed is shown as a small open grey circle overlaying haplotypes of Clade 4 where overlap in ranges was observed. Each haplotype is indicated except for singletons (haplotypes observed only once), which share the same symbol.

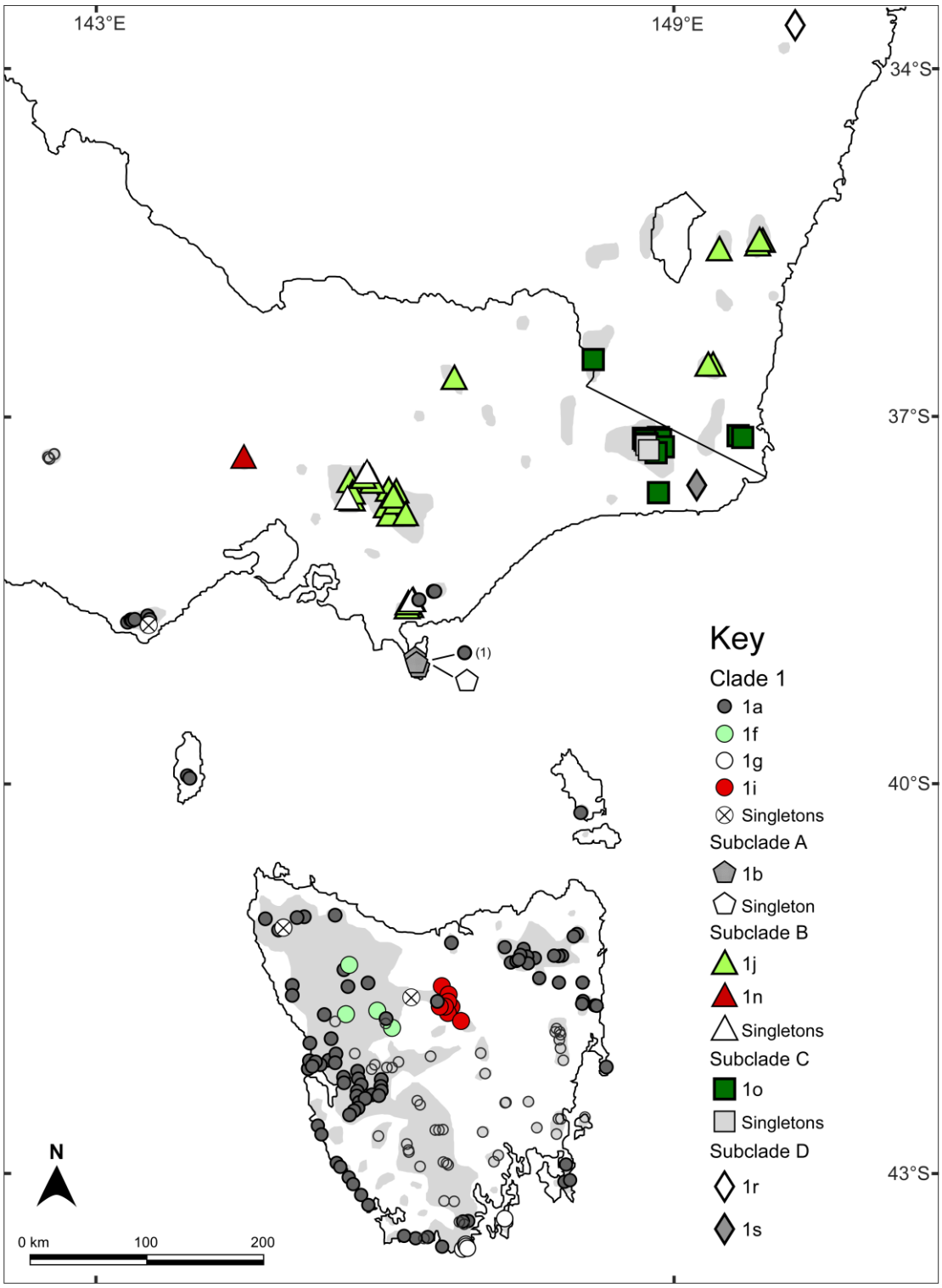


Fig. 8 Distribution of clade 1 haplotypes observed across the range of *Tasmannia lanceolata*. For reference, each sample location bearing haplotypes of other clades is displayed as a small open grey circle. Each haplotype is distinguished except for singletons (haplotypes observed only once), which share the same symbol.

Discussion

Chloroplast evidence for short-range dispersal

The chloroplast phylogeography of *Tasmannia lanceolata* is in direct conflict with all three predictions of the hypothesis that the fleshy fruit of this species has enabled effective long-distance movements across the species range leading to low genetic structuring (see Introduction of this chapter). Firstly, most haplotype clades and subclades are restricted in their range. Hypothetically, such structuring could be the consequence of recent colonisation followed by rapid evolution. However, considering the slow rate of chloroplast evolution (Wolfe *et al.*, 1987; Clegg *et al.*, 1994) and the evidence for a particularly slow rate of molecular evolution in the Winteraceae family (Suh *et al.*, 1993) it is probable that most of the haplotypes are old. Indeed some of the clades are likely to be very old, particularly clades 2, 3 and 4, which are distinct by multiple polymorphisms. The locations of each clade and or subclade are likely to be the consequence of long-term occupation with evidence for *in situ* diversification particularly in western Tasmania and southern Victoria.

Secondly, the chloroplast patterns of *T. lanceolata* are notable for the near absence of admixture of haplotypes. This lack of admixture indicates that the effective dispersal of *T. lanceolata* has been limited, especially in terms of long-distance seed dispersal, despite the presumed high mobility of the species. Effective long-distance dispersal has not occurred frequently enough over time to prevent the development (or to erase) the strong phylogeographic structure observed in large parts of the range of *T. lanceolata*. Rather, the chloroplast evidence indicates that the dispersal of *T. lanceolata,* especially any migration that occurred during the Holocene wet forest expansion ~ 9000 years ago (Macphail, 1979; Sweller & Martin, 2001; McKenzie, 2002) has been dominated by short-range movements, a phenomenon which is well demonstrated in particular by the geographic clustering of many rare haplotypes within clades and subclades. However, some caution must be given as effective long-distance dispersal events may not always be apparent in surveys of haplotype distributions if the source and sink populations were of the same haplotype, or the immigrant haplotypes remain were undetected in the sink population.

Thirdly, fleshy fruitedness of *T. lanceolata* appears to have not enabled frequent effective dispersal across unsuitable habitat into disjunct populations. In multiple cases disjunct populations were found to harbour endemic haplotypes (Mt Macedon

and the Blue Mountains) or endemic subclades or clades (Wilsons Promontory or the Grampians). These results indicate the long-term isolation of these populations that may have been established by either rare long-distance dispersal events or range fragmentation, and the subsequent dominance of regeneration by short-range dispersal.

Overall, the strong phylogeographic structure observed within *T. lanceolata* provides strong evidence for the limited dispersal of this species and events of long-distance dispersal being rare. This unexpected finding requires careful scrutiny of the factors that may be involved, including evidence for the dominance of short-range dispersal, the difficulties in establishing new populations especially at long-distances from source populations and the probable reproductive inertia of *T. lanceolata* populations, particularly in the drier parts of the species range; and possible selection for local genotypes.

Evidence for the dominance of short-range dispersal

Southeastern Australia has few specialist frugivorous bird species (Forde, 1986) and no species that include *T. lanceolata* berries as a major component of their diet. In Tasmania, two endemic birds are known to consume the fruit of *T. lanceolata*, the green rosella (*Platycercus caledonicus*; Psittacidae) and the black currawong (*Strepera fuliginosa*; Cracticidae). Although *Platycercus* species are seed predators (French, 1992), *P. caledonicus* is known to regurgitate intact seed of *T. lanceolata* (probably due to overconsumption; (Read & Hill, 1983)) and is considered the main bird disperser of *T. lanceolata* at low altitudes (Read, 1982; Read & Hill, 1983). The omnivorous *S. fuliginosa* regurgitates intact seed of *T. lanceolata* after stripping of the fleshy covering (Cash, 1998; Borzak, 2003). For example, *T. lanceolata* seed was observed in 8 of 91 (Borzak, 2003) and 4 of 26 (Cash, 1998) regurgitated pellets collected from across Tasmania. On mainland Australia, the birds that consume the fruit of *T. lanceolata* are apparently unknown, although both *Platycercus* and *Strepera* species are common throughout its mainland range (Blakers *et al.*, 1984).

Available evidence indicates that seed dispersal by these birds is likely to be dominated by short-range movements. Large-scale migration is not a feature of temperate Australian birds (French *et al.*, 1992), apparently including both species (e.g. Ratkowsky & Ratkowsky, 1978). Populations of both species on both Flinders and King Islands in Bass Strait, for example, are considered to be separate subspecies

(Schodde & Mason, 1997). However, Wilkinson (1997) considered that the long-distance events of seed dispersal by exploratory movements of birds, particularly young birds, may have greater importance than return migration in its impact on long-distance dispersal of plants. The mainland relative of the *S. fuliginosa*, the pied currawong *S. graculina* (Ridpath & Moreau, 1966), are known to move over 200 km in the Snowy Mountains in the search for new territory when immature (Wimbush, 1969). Therefore, the regurgitation of seed, rather than lack of long-distance movement by the birds, may be a critical factor in limiting *T. lanceolata* dispersal. In fact, the feeding behaviour of *S. graculina* suggests that the majority of seed may be regurgitated 5 to 15 minutes after fruit consumption therefore close to the food source (Bass, 1990).

Two marsupials are also known to consume *T. lanceolata* fruit. (Read & Hill, 1983) observed *T. lanceolata* in scats of brushtail possums (*Trichosurus vulpecula*) and red-bellied wallabies (*Thylogale billardierii*) in a heavy fruiting year. These species have home range up to 40 hectares in size (le Mar, 2002) but any individual movement of the brushtail possum within this home range are unlikely to be greater than ~ 2 km (Clout & Efford, 1984).

Other agents of fruit dispersal are likely to result in short-distance movements in *T. lanceolata*. Firstly, a large proportion of the fruit crop of *T. lanceolata* may not be dispersed by birds or other animals, as has been observed in *Olea europea* with 65% of the total crop not removed in one fruiting season (Rey & Alcantara, 2000), and 50% of fruit not consumed over one fruiting season in *Rubus saxatilis* (Eriksson & Bremer, 1993). These fruits may eventually be dispersed near to the plant by gravity or possibly dislodged by strong winds. Such mechanisms of limited dispersal have been observed as being important in the spatial extent of seed dispersal in other fleshy fruited plants including *Sassafras randaiense* (Guan *et al.*, 2006) and *Ocotenia endresiana* (Wenny, 2000). Once *T. lanceolata* seed reaches the ground it may be secondarily dispersed by ants, as has been observed for *T. lanceolata* seed by (Howard, 1974), or water, as in the fleshy fruited plant *Frangula alnus* subsp. *baetica* (Hampe & Arroyo, 2002). Ant dispersal is generally limited to a few metres (Andersen, 1988; He *et al.*, 2008) while water dispersal does not provide any mechanism to cross catchments.

The difficulty of establishing new populations

Although the dispersal of *T. lanceolata* is likely to be dominated by short-range movements as described above, some long-distance dispersal events are likely to have occurred. Firstly, as with other fleshy fruited plants (Snow, 1970) the fruit of *T. lanceolata* are likely to be consumed by a variety of birds (Read, 1982) with different feeding behaviours. In addition, Higgins *et al.* (2003) described numerous mechanisms that would not be expected based on the morphology of propagules that have been observed to disperse seed or fruit in plants such as sticking to birds feet, ocean currents and turbulent winds.

A number of factors may decrease the probability of successful establishment by rare long-distance dispersal in unoccupied habitat. Firstly, *T. lanceolata* usually occurs in a mosaic of dry and wet habitats so that most seed transported long-distances may fall into dry habitats unsuitable for establishment (Eriksson & Bremer, 1991). Secondly, seed that does disperse to a favourable site by long-distance dispersal events face numerous post-dispersal effects that may prevent successful establishment (Howe, 1989; Houle, 1995). These effects include factors that may prevent germination, seedling and juvenile survival and, finally, survival to reproductive maturity (Wang & Smith, 2002). However, it is uncertain whether the potential for successful establishment of *T. lanceolata* is decreased by the presence of conspecifics. If this is the case then the evidence for the long-term occupation of *T. lanceolata* in multiple regions may be a factor explaining the evidence for the failure of rare long-distance events to have a genetic impact on the phylogeography of the species. Failure of establishment near conspecifics has been observed in a wide-range of studies (e.g. Salomonson, 1978; Howe *et al.*, 1985; Rey & Alcantara, 2000). In addition, the likelihood of any successful long-distance dispersal event having a genetic 'impact' on any established population is lessened due to the fact that the existing population (and its haplotype(s)) is likely to dominate the seed in the canopy and the *T. lanceolata* soil seed bank that has been observed by Howard (1974), where seed may survive for some years (Read, 1999). Therefore, any large-scale disturbance or 'canopy gap' sexual regeneration of this species is most likely to be dominated by seed possessing the haplotype(s) of the invaded population.

A third factor that may cause difficulty in establishing new *T. lanceolata* populations via long-distance dispersal may be the dioecy of the species. This means that the

successful establishment of a new population via a single seed event is difficult because it depends on the seed being female, and subsequent availability of pollen. This effect would be particularly exacerbated at sites that are far from seed sources where individual seed dispersal events are likely to be rare and plants of the opposite sex are not already in the vicinity.

The strong phylogeographic structure of *T. lanceolata* and in particular the strong evidence for a limited distance of migration during the Holocene could partly be explained by the Allee effect. The Allee effect includes any positive relationships between the density or numbers of a species and individual fitness (Stephens *et al.*, 1999), that results in decreased reproduction and survival of individuals. In plants Allee effects can be associated with low population density, small patch size and patch isolation and are thought to be particularly predominant in insect pollinated plants (Groom, 1998), such as *T. lanceolata*. Allee effects are thought to be able to constrain both population growth and prevent the establishment of new populations, therefore decreasing the migration ability of species (Tobin *et al.*, 2007). Such demographic 'inertia' may explain the succinct boundary between the southeastern Tasmanian clade (clade 2) and the western and southern Tasmanian clade (clade 4). Populations of *T. lanceolata* in the western half of the distribution of clade 2 are widely scattered, presumably because of the dominance of an unsuitable dry climate and high fire frequency. In this landscape where suitable microsites for *T. lanceolata* are strongly patchily distributed, Allee effects may prevent the populations exceeding critical thresholds of propagule output (Grime, 2002). This would result in poor effective dispersal of seed bearing haplotypes of both clades into this region, allowing the development and/or maintenance of the boundary between these haplotypes (Keitt *et al.*, 2001). However, Allee effects are less likely to be involved in the boundaries in western Tasmania between haplotypes of clades 1 and 4 where *T. lanceolata* populations are generally large and continuous.

The possible role of selection

One possible explanation for the strong chloroplast patterns observed in *T. lanceolata* may be selection for local genotypes. A case in point is the geneological boundaries between clade 1 and 4 in western Tasmania (Fig. 6). These may have arisen through poor effective dispersal alone and therefore could be viewed as being a 'snapshot' in time of two slowly expanding fronts (Neigel & Avise, 1986). However, the near

absence of admixture of haplotypes from these two clades is remarkable. All tests for selection acting on the chloroplast sequences were non-significant. This coupled with the absence of recombination in the chloroplast genome (Ennos *et al.*, 1999) suggests that it is unlikely that any selection for particular cpDNA mutation can be implicated in the observed chloroplast patterns. However, different chloroplast haplotypes may be associated with divergent nuclear genomes. Therefore, selection against the nuclear genome of immigrants because of maladaptation may be considered in explaining the geneological boundaries within *T. lanceolata*. In a study of the tropical rainforest tree *Cedrela odorata* a similar geneological boundary between cpDNA lineages was observed within the continuous range of this species in central America, concomitant with the distribution of two distinct ecotypes of the species differing significantly both morphologically and physiologically (Cavers *et al.*, 2003). The western Tasmanian boundary observed in *T. lanceolata* does not coincide with the distribution of the two stomatal forms observed by Barnes *et al.* (2000) (Fig. 2). In addition, the environmental heterogeneity of factors such as pH, humidity and geology are likely to be at least as significant within the area occupied by each clade in Tasmania as the heterogeneity between the areas occupied by the different clades.

Other implications

The locations of the refugia implicit in the distribution of *T. lanceolata* haplotypes are in broad agreement with previous pollen and molecular based evidence for the glacial refugia of the rainforest tree *Nothofagus cunninghamii* within western Tasmania and the central highlands of Victoria (McKenzie, 1997; Colhoun, 2000; Chapter 2). The *T. lanceolata* patterns also provide the first genetic based evidence for a glacial refugium of a mesic plant species postulated for Wilsons Promontory (Ladd, 1979a, 1991). This study also provides additional evidence for glacial refugia of mesic plants in east Gippsland (Hope, 1984; Kershaw *et al.*, 1986; Nevill *et al.*, 2008). This study also observed strong evidence for long-term survival in some unexpected locations, such as in southeastern Tasmania where clade 2 haplotypes were observed. This was unexpected for two main reasons, firstly the major cool temperate rainforest tree *N. cunninghamii* is absent from this area (apart from a very small stand at Yarlington Tier). Second, no rainforest or *Eucalyptus* wet forest refugia were inferred in this area at the height of the Last Glacial Maximum (Kirkpatrick & Fowler, 1998). However, the area is the location of two distinctive chloroplast lineages in *Eucalyptus* subgenus *Symphomyrtus* (Freeman *et al.*, 2001; McKinnon *et al.*, 2004). The

occurrence of a distinct clade (clade 3) in the Grampians provides strong evidence isolation of this population through at least the Last Glacial Maximum. This finding is perhaps consistent with the occurrence of endemic plant taxa (Crisp *et al.*, 2001) in the Grampians including endemic subspecies of some wet forest species such as *Prostanthera lasianthos* and *Pomaderris apetala* that, like *T. lanceolata*, also have their most westerly occurrence in the Grampians.

The large chloroplast divergence of *Tasmannia* from the other Winteraceae representatives *Pseudowintera* and *Drimys* is the strongest molecular evidence to date for the distinctiveness of *Tasmannia* within the Winteraceae and builds upon a substantial body of morphological, karyological and phylogenetic evidence (Ehrendorfer *et al.*, 1968; Smith, 1969; Suh *et al.*, 1993; Karol *et al.*, 2000; Doust & Drinnan, 2004). In addition, the observation of two strongly supported cpDNA lineages of *Tasmannia* within Australia, one that is composed entirely of the mainly southern distributed *T. lanceolata*, and the other comprising all other Australian species investigated (except *T. membranea*, which is not included in this study) is consistent with observations that *T. lanceolata* is distinctive morphologically from other members of the genus (Vink, 1970) and is the most morphologically diverged among the Australian species (Sampson *et al.*, 1988).

Conclusions

This study has provided an important contribution to understanding the consequences of fleshy fruitedness on the effective dispersal and the genetic structure of plants. The strong phylogeographic structure found in *T. lanceolata*, at both the large-scale (i.e range-wide) and at a small spatial scale, particularly in Tasmania, demonstrate that the fleshy-fruitedness and bird-dispersal of this species have not resulted in extensive effective seed flow at long-distances. Rather the species has maintained long-term presence in multiple parts of its range due to establishment via short-distance movements of seed. The underlying causes of this phenomenon appear to be related to the absence of any vector of dispersal that could provide persistent movement of seed across the patchy distribution of this species. Any long-distance movements by the two bird species that are known to consume the fruit have not been sufficient to prevent the development of strong phylogeographic structuring, possibly due to their quick regurgitation of the seed. In addition, factors such as competition with conspecifics, dioecy, and/ or selection may lead to high

post-dispersal establishment failure in this species. The findings of this study have some important implications. Firstly, they re-enforce the view that propagule traits can be poor predictors of the mobility of a plant and their genetic structure. Secondly, this study demonstrates that many factors need to be considered when predicting the potential for long-distance dispersal in plants, including bird behaviour and post-dispersal factors. Thirdly, this study indicates that fleshy-fruited and bird-dispersed plants can display strong genetic signals of past climate and glacial refugia. Further genetic studies of fleshy fruited and bird-dispersed plants in Australia and in other parts of the world are required.

Appendices

Appendix 1

Tasmannia lanceolata sample information for the range-wide chloroplast study (244 samples), including the haplotype and clade/subclade of each sample.

N	Location	Region	State	Lat.	Long.	Alt. (masl)	Haplo -type	Clade/ subclade
1	Wallaby Creek Rd.	Dazzler Ranges	Tasmania	-41.269	146.67	121	1a	Clade 1
2	Saxons Creek	Dazzler Ranges	Tasmania	-41.27	146.67	105	1a	Clade 1
3	Lookout Hill Rainforest Ledge	Douglas-Apsley NP	Tasmania	-41.741	148.227	387	1a	Clade 1
4	Douglas River	Douglas-Apsley NP	Tasmania	-41.735	148.205	364	1a	Clade 1
5	Mt Strzelecki	Flinders Island	Tasmania	-40.205	148.062	750	1a	Clade 1
6	Mt Strzelecki	Flinders Island	Tasmania	-40.205	148.062	768	1a	Clade 1
7	Graham Creek	Freycinet Peninsula	Tasmania	-42.206	148.329	450	1a	Clade 1
8	Graham Creek	Freycinet Peninsula	Tasmania	-42.206	148.33	465	1a	Clade 1
9	Huntsman Road	Great Western Tiers	Tasmania	-41.706	146.595	410	1a	Clade 1
10	Fraser River	King Island	Tasmania	-39.939	144.018	90	1a	Clade 1
11	Sea Elephant River	King Island	Tasmania	-39.923	143.986	71	1a	Clade 1
12	Horseshoe Marsh near St Pauls River	Mt Puzzler/ Fingal Tier	Tasmania	-41.709	148.094	551	1a	Clade 1
13	near tributory of St Pauls River	Mt Puzzler/Fingal Tier	Tasmania	-41.695	148.088	659	1a	Clade 1
14	Northallerton Road	North East Tasmania	Tasmania	-41.395	147.547	507	1a	Clade 1
15	Ben Ridge Road	North East Tasmania	Tasmania	-41.361	147.547	795	1a	Clade 1
16	Blue Tier	North East Tasmania	Tasmania	-41.192	148.005	745	1a	Clade 1
17	Blue Tier	North East Tasmania	Tasmania	-41.169	148.008	682	1a	Clade 1
18	Weavers Creek Road	North East Tasmania	Tasmania	-41.393	147.363	903	1a	Clade 1
19	Mt Albert Rd	North East Tasmania	Tasmania	-41.346	147.843	843	1a	Clade 1

20	Ben Lomond	North East Tasmania	Tasmania	-41.507	147.651	1163	1a	Clade 1
21	Mt Albert summit	North East Tasmania	Tasmania	-41.349	147.872	1090	1a	Clade 1
22	Mount Arthur	North East Tasmania	Tasmania	-41.277	147.289	1100	1a	Clade 1
23	Mount Barrow-low	North East Tasmania	Tasmania	-41.358	147.426	783	1a	Clade 1
24	Mount Barrow-high	North East Tasmania	Tasmania	-41.379	147.426	1310	1a	Clade 1
25	Myrtle Grove Plantation	North East Tasmania	Tasmania	-41.289	147.492	611	1a	Clade 1
26	St Patricks River	North East Tasmania	Tasmania	-41.317	147.465	546	1a	Clade 1
27	Ben Ridge Road	North East Tasmania	Tasmania	-41.366	147.613	894	1a	Clade 1
28	Mt Victoria	North East Tasmania	Tasmania	-41.34	147.824	809	1a	Clade 1
29	East Tower, Tower Hill	North East Tasmania	Tasmania	-41.547	147.861	913	1a	Clade 1
30	East Tower, Tower Hill	North East Tasmania	Tasmania	-41.547	147.853	1124	1a	Clade 1
31	Mt Durham, Nicholas Range	North East Tasmania	Tasmania	-41.55	148.076	611	1a	Clade 1
32	Ocean Rd. near Stalker	Otway Ranges	Victoria	-38.665	143.416	616	1a	Clade 1
33	Ocean Rd. near Stalker	Otway Ranges	Victoria	-38.665	143.415	611	1a	Clade 1
34	McKenzie Rd.	Otway Ranges	Victoria	-38.654	143.417	395	1a	Clade 1
35	McKenzie Rd.	Otway Ranges	Victoria	-38.664	143.418	517	1a	Clade 1
36	Binns Rd., near Beech Forest	Otway Ranges	Victoria	-38.647	143.587	503	1a	Clade 1
37	Aire Valley Plantation, Binns Rd.	Otway Ranges	Victoria	-38.684	143.582	437	1a	Clade 1
38	Aire Valley Plantation, Binns Rd.	Otway Ranges	Victoria	-38.69	143.593	505	1a	Clade 1
39	Chappel Creek, near Lavers Hill	Otway Ranges	Victoria	-38.681	143.386	429	1a	Clade 1
40	Albert River	Strzelecki Ranges	Victoria	-38.508	146.401	334	1a	Clade 1
41	Tarra Bulga NP, Mack Creek	Strzelecki Ranges	Victoria	-38.428	146.571	601	1a	Clade 1
42	Tarra Bulga NP, carpark	Strzelecki Ranges	Victoria	-38.43	146.566	658	1a	Clade 1
43	Waterfall Bay	Tasman Peninsula	Tasmania	-43.066	147.947	190	1a	Clade 1
44	Tatnells Hill	Tasman Peninsula	Tasmania	-43.084	147.923	463	1a	Clade 1
45	MacGregors Peak	Tasman Peninsula	Tasmania	-42.976	147.931	427	1a	Clade 1

46	Black Bluff Range (Rocky Mount Lookout)	Western Tasmania	Tasmania	-41.539	145.871	968	1a	Clade 1
47	New Pelion Hut	Western Tasmania	Tasmania	-41.827	146.051	790	1a	Clade 1
48	near Henty River, Zeehan Hwy	Western Tasmania	Tasmania	-41.99	145.492	311	1a	Clade 1
49	Bulgobac River	Western Tasmania	Tasmania	-41.582	145.678	669	1a	Clade 1
50	Fossey River	Western Tasmania	Tasmania	-41.449	145.619	618	1a	Clade 1
51	Pieman River	Western Tasmania	Tasmania	-41.654	145.078	23	1a	Clade 1
52	The Longback, near Donaldson River	Western Tasmania	Tasmania	-41.564	145.088	295	1a	Clade 1
53	near Edith Creek	Western Tasmania	Tasmania	-41.136	144.951	81	1a	Clade 1
54	Wedge Plains Rd.	Western Tasmania	Tasmania	-41.032	145.213	197	1a	Clade 1
55	Harry Ryan Creek	Western Tasmania	Tasmania	-41.043	145.124	182	1a	Clade 1
56	McDougalls Hill	Western Tasmania	Tasmania	-43.577	146.888	12	1a	Clade 1
57	Hot Springs Creek, near Hastings Cave	Western Tasmania	Tasmania	-43.391	146.85	94	1a	Clade 1
58	Creekton Rivulet	Western Tasmania	Tasmania	-43.375	146.893	107	1a	Clade 1
59	Henty Bridge	Western Tasmania	Tasmania	-42.023	145.267	20	1a	Clade 1
60	near Rosebery	Western Tasmania	Tasmania	-41.805	145.421	220	1a	Clade 1
61	Macquarie Heads Rd. Swan Basin	Western Tasmania	Tasmania	-42.205	145.267	5	1a	Clade 1
62	Ocean Beach Rd.	Western Tasmania	Tasmania	-42.148	145.265	10	1a	Clade 1
63	Macquarie Heads Rd. Swan Basin	Western Tasmania	Tasmania	-42.214	145.27	20	1a	Clade 1
64	Teepookana	Western Tasmania	Tasmania	-42.194	145.365	5	1a	Clade 1
65	Purgatory Creek	Western Tasmania	Tasmania	-42.289	145.614	320	1a	Clade 1
66	Lyell Hwy, Starting Creek	Western Tasmania	Tasmania	-42.148	145.464	225	1a	Clade 1
67	Woody Hill	Western Tasmania	Tasmania	-42.148	145.466	220	1a	Clade 1
68	Hogarth Falls track	Western Tasmania	Tasmania	-42.153	145.338	25	1a	Clade 1
69	Newell Creek	Western Tasmania	Tasmania	-42.164	145.542	140	1a	Clade 1

70	Purgatory Creek	Western Tasmania	Tasmania	-42.307	145.613	210	1a	Clade 1
71	South Queenstown	Western Tasmania	Tasmania	-42.1	145.539	120	1a	Clade 1
72	Newell Creek	Western Tasmania	Tasmania	-42.164	145.538	100	1a	Clade 1
73	Franklin River Rd., near Nora River	Western Tasmania	Tasmania	-42.316	145.619	210	1a	Clade 1
74	Mine Creek, Cox Bight	Western Tasmania	Tasmania	-43.49	146.246	10	1a	Clade 1
75	Ironbound Range east	Western Tasmania	Tasmania	-43.513	146.467	715	1a	Clade 1
76	Louisa Beach	Western Tasmania	Tasmania	-43.515	146.358	10	1a	Clade 1
77	Surprise Bay West	Western Tasmania	Tasmania	-43.578	146.646	55	1a	Clade 1
78	Detention Falls Rd.	Western Tasmania	Tasmania	-41.016	145.539	239	1a	Clade 1
79	Warra Rd.	Western Tasmania	Tasmania	-41.046	144.819	79	1a	Clade 1
80	Endeavour Bay	Western Tasmania	Tasmania	-42.652	145.342	8	1a	Clade 1
81	Christmas Cove	Western Tasmania	Tasmania	-42.724	145.392	10	1a	Clade 1
82	Lewis River	Western Tasmania	Tasmania	-42.945	145.552	41	1a	Clade 1
83	Cowrie Beach	Western Tasmania	Tasmania	-42.972	145.558	22	1a	Clade 1
84	confluence of Collingwood and Franklin Rvs	Western Tasmania	Tasmania	-42.2	145.931	308	1a	Clade 1
85	Finchams Crossing, Franklin River	Western Tasmania	Tasmania	-42.242	145.767	219	1a	Clade 1
86	Camp Arcade, Franklin River	Western Tasmania	Tasmania	-42.285	145.747	231	1a	Clade 1
87	Coruscades, Franklin River	Western Tasmania	Tasmania	-42.329	145.793	181	1a	Clade 1
88	Rafters Basin, Franklin River	Western Tasmania	Tasmania	-42.365	145.772	113	1a	Clade 1
89	Newlands Cascade, Franklin River	Western Tasmania	Tasmania	-42.422	145.756	124	1a	Clade 1
90	Blackmans Bend, Franklin River	Western Tasmania	Tasmania	-42.517	145.768	26	1a	Clade 1
91	Sir John Falls, Gordon River	Western Tasmania	Tasmania	-42.57	145.69	21	1a	Clade 1
92	Adelaide River	Western Tasmania	Tasmania	-42.297	146.016	471	1a	Clade 1
93	Erebus Rivulet	Western Tasmania	Tasmania	-42.387	145.995	298	1a	Clade 1

94	Jane River	Western Tasmania	Tasmania	-42.413	145.979	280	1a	Clade 1
95	Jane River	Western Tasmania	Tasmania	-42.422	145.909	183	1a	Clade 1
96	Gilgamesh Gorge, Jane River	Western Tasmania	Tasmania	-42.455	145.83	118	1a	Clade 1
97	Humbaba Gorge, Jane River	Western Tasmania	Tasmania	-42.448	145.788	70	1a	Clade 1
98	Nye Bay	Western Tasmania	Tasmania	-43.052	145.677	8	1a	Clade 1
99	Mulcahy River	Western Tasmania	Tasmania	-43.107	145.716	22	1a	Clade 1
100	Wreck Bay	Western Tasmania	Tasmania	-43.191	145.793	14	1a	Clade 1
101	Kelly Basin	Western Tasmania	Tasmania	-43.271	145.871	10	1a	Clade 1
102	Mt Latrobe	Wilsons Promontory	Victoria	-39.004	146.377	744	1a	subclade A
103	Mt Ramsay	Wilsons Promontory	Victoria	-39.032	146.381	413	1b	subclade A
104	Mt Ramsay	Wilsons Promontory	Victoria	-39.031	146.382	399	1b	subclade A
105	Mt Ramsay	Wilsons Promontory	Victoria	-39.024	146.383	680	1b	subclade A
106	Mt Ramsay	Wilsons Promontory	Victoria	-39.02	146.38	647	1b	subclade A
107	Mt Latrobe	Wilsons Promontory	Victoria	-39.009	146.376	488	1b	subclade A
108	Mt Latrobe	Wilsons Promontory	Victoria	-39.002	146.377	786	1c	subclade A
109	Arthur River	Western Tasmania	Tasmania	-41.115	144.984	61	Id	Clade 1
110	Devils Gullet	Western Tasmania	Tasmania	-41.664	146.322	1152	Ie	Clade 1
111	Fergusson Falls	Western Tasmania	Tasmania	-41.903	146.122	810	1f	Clade 1
112	Windermere Hut	Western Tasmania	Tasmania	-41.772	145.954	1009	1f	Clade 1
113	Mount Murchison	Western Tasmania	Tasmania	-41.798	145.635	404	1f	Clade 1
114	Hellyer River	Western Tasmania	Tasmania	-41.416	145.674	566	1f	Clade 1
115	Mount Mangana	Bruny Island	Tasmania	-43.361	147.287	450	1g	Clade 1
116	Cockle Creek	Western Tasmania	Tasmania	-43.58	146.899	2	1g	Clade 1
117	Heather Creek	Western Tasmania	Tasmania	-43.597	146.875	15	1g	Clade 1
118	Catamaran River	Western Tasmania	Tasmania	-43.557	146.884	9	1g	Clade 1
119	Aire Valley Plantation, Binns Rd.	Otway Ranges	Victoria	-38.687	143.585	475	1h	Clade 1

120	Dunning rivulet	Great Western Tiers	Tasmania	-41.737	146.612	600	1i	Clade 1
121	Long Back Road	Great Western Tiers	Tasmania	-41.587	146.63	221	1i	Clade 1
122	Breona	Great Western Tiers	Tasmania	-41.777	146.708	1074	1i	Clade 1
123	Upper Liffey Road	Great Western Tiers	Tasmania	-41.733	146.712	1133	1i	Clade 1
124	Lake Highway, near Halfmoon Creek	Great Western Tiers	Tasmania	-41.756	146.713	1150	1i	Clade 1
125	Quamby Bluff	Great Western Tiers	Tasmania	-41.654	146.696	1193	1i	Clade 1
126	Poatina Rd, east side of Great Lake	Western Tasmania	Tasmania	-41.854	146.847	1099	1i	Clade 1
127	Projection Bluff	Western Tasmania	Tasmania	-41.725	146.719	1258	1i	Clade 1
128	Brown Mountain	Brown Mountain	NSW	-36.601	149.441	899	1j	subclade B
129	Brown Mountain	Brown Mountain	NSW	-36.594	149.444	856	1j	subclade B
130	Brown Mountain	Brown Mountain	NSW	-36.6	149.378	1240	1j	subclade B
131	Icy Creek	Central Highlands Vic	Victoria	-37.859	146.123	527	1j	subclade B
132	Tanjil River, East Tanjil Road	Central Highlands Vic	Victoria	-37.832	146.193	507	1j	subclade B
133	East Tanjil Road	Central Highlands Vic	Victoria	-37.837	146.198	549	1j	subclade B
134	headwaters of Hope Creek, East Tanjil Rd.	Central Highlands Vic	Victoria	-37.849	146.253	1218	1j	subclade B
135	Toorongo Road, near trib of Mundic Creek	Central Highlands Vic	Victoria	-37.785	146.064	822	1j	subclade B
136	Toorongo Road	Central Highlands Vic	Victoria	-37.789	146.097	959	1j	subclade B
137	Myrrhee	Central Highlands Vic	Victoria	-37.77	146.147	1093	1j	subclade B
138	headwaters of Upper Thomson River	Central Highlands Vic	Victoria	-37.745	146.161	1074	1j	subclade B
139	headwaters of Upper Thomson River	Central Highlands Vic	Victoria	-37.72	146.152	1088	1j	subclade B
140	headwaters of Shaw Creek	Central Highlands Vic	Victoria	-37.633	146.008	1008	1j	subclade B
141	Mt Morgan, near Cumberland Junction	Central Highlands Vic	Victoria	-37.565	145.905	936	1j	subclade B

142	headwaters of Oaks Creek, nr. The Triangle	Central Highlands Vic	Victoria	-37.652	146.11	1064	1j	subclade B
143	Mt Donna Buang Rainforest Gallery Walk	Central Highlands Vic	Victoria	-37.712	145.703	720	1j	subclade B
144	Archeron Way, near Somers Park	Central Highlands Vic	Victoria	-37.636	145.713	575	1j	subclade B
145	Acheron Way, near St Fillians	Central Highlands Vic	Victoria	-37.565	145.682	471	1j	subclade B
146	Lake Mountain, near summit	Central Highlands Vic	Victoria	-37.498	145.877	1400	1j	subclade B
147	Ythan Creek	Central Highlands Vic	Victoria	-37.722	145.683	1051	1j	subclade B
148	Snowy Junction, Cumberland Rd.	Central Highlands Vic	Victoria	-37.534	145.835	1008	1j	subclade B
149	Clyde Mountain	Monga NP	NSW	-35.551	149.953	713	1j	subclade B
150	Mongarlowe River	Monga NP	NSW	-35.562	149.919	655	1j	subclade B
151	Mongarlowe River	Monga NP	NSW	-35.601	149.913	705	1j	subclade B
152	McKinnon's Corner	Mt Buffalo	Victoria	-36.716	146.785	1246	1j	subclade B
153	Mt Fatigue, near Falls Creek	Strzelecki Ranges	Victoria	-38.583	146.312	294	1j	subclade B
154	Mt Fatigue, near Falls Creek	Strzelecki Ranges	Victoria	-38.583	146.31	345	1j	subclade B
155	Mt Fatigue	Strzelecki Ranges	Victoria	-38.575	146.3	454	1j	subclade B
156	headwaters of Agnes River	Strzelecki Ranges	Victoria	-38.558	146.315	453	1j	subclade B
157	Tallaganda NP	Tallaganda NP	NSW	-35.638	149.506	1071	1j	subclade B
158	Mt Donna Buang near summit	Central Highlands Vic	Victoria	-37.714	145.673	1242	1k	subclade B
159	Snowy Hill	Central Highlands Vic	Victoria	-37.524	145.847	1111	1l	subclade B
160	The Grand Ridge Rd, headwaters of Agnes Rv	Strzelecki Ranges	Victoria	-38.534	146.331	486	1m	subclade B
161	Mt Macedon	Mt Macedon	Victoria	-37.372	144.59	924	1n	subclade B
162	Mt Macedon	Mt Macedon	Victoria	-37.378	144.577	982	1n	subclade B
163	Mt Macedon	Mt Macedon	Victoria	-37.374	144.581	941	1n	subclade B
164	Bemm River	Bemm River	Victoria	-37.632	148.886	44	1o	subclade C
165	Bemm River	Bemm River	Victoria	-37.633	148.885	45	1o	subclade C
166	Gap Rd/ Orbost Bonang Rd junction	Errinundra Plateau	Victoria	-37.244	148.757	752	1o	subclade C
167	Bonang River	Errinundra Plateau	Victoria	-37.232	148.754	736	1o	subclade C

168	Bonang River	Errinundra Plateau	Victoria	-37.209	148.74	712	1o	subclade C
169	Bonang River	Errinundra Plateau	Victoria	-37.207	148.73	700	1o	subclade C
170	Clarksville Rd.	Errinundra Plateau	Victoria	-37.223	148.875	937	1o	subclade C
171	Clarksville Rd.	Errinundra Plateau	Victoria	-37.235	148.893	1051	1o	subclade C
172	Goonmirk Rocks Rd.	Errinundra Plateau	Victoria	-37.252	148.906	1114	1o	subclade C
173	Goonmirk Rocks	Errinundra Plateau	Victoria	-37.276	148.885	1219	1o	subclade C
174	Goonmirk Rocks Rd.	Errinundra Plateau	Victoria	-37.276	148.889	1201	1o	subclade C
175	Goonmirk Rocks Rd.	Errinundra Plateau	Victoria	-37.274	148.891	1195	1o	subclade C
176	tributory of Leather Barrel Creek	Kosciusko NP, NSW	NSW	-36.532	148.198	1150	1o	subclade C
177	tributory of Leather Barrel Creek	Kosciusko NP, NSW	NSW	-36.532	148.198	1150	1o	subclade C
178	tributory of Leather Barrel Creek	Kosciusko NP, NSW	NSW	-36.532	148.198	1150	1o	subclade C
179	Mt Imlay	Mt Imlay NP, NSW	NSW	-37.182	149.731	865	1o	subclade C
180	Mt Imlay	Mt Imlay NP, NSW	NSW	-37.182	149.731	870	1o	subclade C
181	Mt Imlay	Mt Imlay NP, NSW	NSW	-37.101	149.872	887	1o	subclade C
182	Gap Road	Errinundra Plateau	Victoria	-37.232	148.806	928	1p	subclade C
183	The Gap Scenic Reserve	Errinundra Plateau	Victoria	-37.249	148.773	813	1q	subclade C
184	Popes Glen Blackheath	Blue Mountains	NSW	-33.632	150.296	1020	1r	subclade D
185	Popes Glen Blackheath	Blue Mountains	NSW	-33.632	150.296	1020	1r	subclade D
186	Drummer Rainforest Walk	Thurra River	Victoria	-37.57	149.271	127	1s	subclade D
187	Drummer Rainforest Walk	Thurra River	Victoria	-37.569	149.271	127	1s	subclade D
188	Meetus Falls, Cygnet River	Eastern Tiers	Tasmania	-41.952	147.885	498	2a	Clade 2
189	Ferrars Tier near Lake Leake	Eastern Tiers	Tasmania	-41.999	147.859	690	2a	Clade 2
190	Brushy River, upper catchment	Eastern Tiers	Tasmania	-41.947	147.85	750	2a	Clade 2
191	Cygnet River headwaters	Eastern Tiers	Tasmania	-41.929	147.848	785	2a	Clade 2
192	Snow Hill	Eastern Tiers	Tasmania	-41.921	147.842	845	2a	Clade 2

193	Lost Falls Lookout	Eastern Tiers	Tasmania	-42.044	147.89	544	2a	Clade 2
194	Big Sassy Creek	Eastern Tiers	Tasmania	-42.152	147.899	482	2a	Clade 2
195	Bishop and Clerk	Maria Island	Tasmania	-42.59	148.108	385	2a	Clade 2
196	Bishop and Clerk	Maria Island	Tasmania	-42.592	148.114	550	2a	Clade 2
197	Mt Ponsonby	South east Tasmania	Tasmania	-42.478	147.538	660	2a	Clade 2
198	Mt Ponsonby	South east Tasmania	Tasmania	-42.476	147.533	714	2a	Clade 2
199	Ringarooma creek, Mt Morrison	South east Tasmania	Tasmania	-42.677	147.627	216	2a	Clade 2
200	Yarlington Tier	South east Tasmania	Tasmania	-42.495	147.3	670	2a	Clade 2
201	Yarlington Tier	South east Tasmania	Tasmania	-42.474	147.301	720	2a	Clade 2
202	Dennistoun Road	South east Tasmania	Tasmania	-42.258	147.102	650	2a	Clade 2
203	Three thumbs 1st saddle	Weilangta	Tasmania	-42.606	147.889	221	2a	Clade 2
204	Three Thumbs last saddle	Weilangta	Tasmania	-42.605	147.865	490	2a	Clade 2
205	Sandspit River	Weilangta	Tasmania	-42.725	147.837	380	2a	Clade 2
206	Alma Tier	Western Tasmania	Tasmania	-42.107	147.047	1009	2a	Clade 2
207	Meetus Falls , Cygnet River	Eastern Tiers	Tasmania	-41.953	147.885	496	2b	Clade 2
208	Cygnet River	Eastern Tiers	Tasmania	-41.944	147.873	650	2b	Clade 2
209	Stockyard Gap	The Grampians	Victoria	-37.347	1422.56	998	3a	Clade 3
210	Stockyard Gap	The Grampians	Victoria	-37.347	142.559	955	3a	Clade 3
211	Stockyard Gap	The Grampians	Victoria	-37.351	142.555	841	3a	Clade 3
212	Boundary Gap	The Grampians	Victoria	-37.317	142.595	781	3a	Clade 3
213	Boundary Gap	The Grampians	Victoria	-37.318	142.594	767	3b	Clade 3
214	Boundary Gap	The Grampians	Victoria	-37.317	142.594	770	3b	Clade 3
215	Platform Peak	South east Tasmania	Tasmania	-42.701	147.062	730	4a	Clade 4
216	Mt Wellington	Western Tasmania	Tasmania	-42.89	147.22	1161	4a	Clade 4
217	Mt Read	Western Tasmania	Tasmania	-41.845	145.542	1124	4a	Clade 4
218	Hartz Mountains, near Ladies Tarn	Western Tasmania	Tasmania	-43.238	146.769	1035	4a	Clade 4
219	Mt Eliza	Western Tasmania	Tasmania	-42.963	146.404	1153	4a	Clade 4

220	Mt Wedge	Western Tasmania	Tasmania	-42.845	146.292	942	4a	Clade 4
221	Mount Ossa summit	Western Tasmania	Tasmania	-41.872	146.041	1580	4a	Clade 4
222	Snowy South	Western Tasmania	Tasmania	-42.942	146.659	1397	4a	Clade 4
223	Lake Skinner track	Western Tasmania	Tasmania	-42.945	146.699	705	4a	Clade 4
224	Little Navarre River	Western Tasmania	Tasmania	-42.169	146.185	753	4a	Clade 4
225	Hastings Cave	Western Tasmania	Tasmania	-43.385	146.841	136	4a	Clade 4
226	Chestermans Road	Western Tasmania	Tasmania	-43.391	146.858	145	4a	Clade 4
227	Ironbound Range west	Western Tasmania	Tasmania	-43.506	146.432	690	4a	Clade 4
228	Cathedral Rock	Western Tasmania	Tasmania	-42.94	147.192	740	4a	Clade 4
229	Griffiths Creek, Lyell Hwy.	Western Tasmania	Tasmania	-42.213	146.092	779	4a	Clade 4
230	Mt Field NP	Western Tasmania	Tasmania	-42.679	146.63	1054	4b	Clade 4
231	Lake Dobson	Western Tasmania	Tasmania	-42.683	146.59	1034	4b	Clade 4
232	Wylds Craig track	Western Tasmania	Tasmania	-42.48	146.422	820	4b	Clade 4
233	Snowy South	Western Tasmania	Tasmania	-42.938	146.675	1151	4b	Clade 4
234	Mt Field NP	Western Tasmania	Tasmania	-42.681	146.581	1250	4c	subclade E
235	Serpentine River, near Bronte Park	Western Tasmania	Tasmania	-42.119	146.517	648	4c	subclade E
236	Double Barrel Creek, Lyell Hwy	Western Tasmania	Tasmania	-42.193	145.948	403	4c	subclade E
237	Nelson River	Western Tasmania	Tasmania	-42.104	145.737	358	4c	subclade E
238	Mt Arrowsmith summit	Western Tasmania	Tasmania	-42.211	146.075	980	4c	subclade E
239	Angel Rain, Franklin River	Western Tasmania	Tasmania	-42.218	145.906	316	4c	subclade E
240	Wylds Craig summit	Western Tasmania	Tasmania	-42.475	146.39	1336	4d	subclade E
241	near Lake Gordon	Western Tasmania	Tasmania	-42.811	146.272	338	4e	Clade 4
242	Mt Wedge track	Western Tasmania	Tasmania	-42.837	146.279	378	4e	Clade 4
243	Lake Gordon	Western Tasmania	Tasmania	-42.742	145.981	276	4f	Clade 4
244	Farmhouse Creek	Western Tasmania	Tasmania	-43.233	146.667	187	4g	Clade 4

Appendix 2

Genbank accession numbers for all haplotype sequence variants obtained in *Tasmannia lanceolata*, other *Tasmannia*, and outgroups, *Pseudowintera colorata* and *Drimys winteri*.

Chloroplast region	Taxa	Genbank accession
*trn*L intron	*T. lanceolata*	FJ786914–FJ786945
	Other *Tasmannia*	FJ786946–FJ786955
	P. colorata	FJ786956
	D. winteri	FJ786957
*trn*L-*trn*F	*T. lanceolata*	FJ786871-FJ786902
	Other *Tasmannia*	FJ786903-FJ786910 and FJ786838
	P. colorata	FJ786912
	D. winteri	FJ786913
*psb*M-*trn*D	*T. lanceolata*	FJ786839-FJ786870
	Other *Tasmannia*	FJ786782-FJ786791
	P. colorata	FJ786792
	D. winteri	FJ786793
*pet*N-*psb*M	*T. lanceolata*	FJ786794-FJ786825
	Other *Tasmannia*	FJ786826-FJ786835
	P. colorata	FJ786836
	D. winteri	FJ786837
*mat*K6-K2	*T. lanceolata*	FJ786738-FJ786769
	Other *Tasmannia*	FJ786770-FJ786779
	P. colorata	FJ786780
	D. winteri	FJ786781
K1-*mat*K1	*T. lanceolata*	FJ786694-FJ786725
	Other *Tasmannia*	FJ786726-FJ786735
	P. colorata	FJ786736
	D. winteri	FJ786737

Chapter 4: Chloroplast phylogeography of the wind-dispersed cool temperate rainforest tree *Atherosperma moschatum* (Atherospermataceae)

Introduction

Worldwide, most areas of forest vegetation in the temperate zone are believed to have been established during the Holocene, largely through expansion from refugia in which they survived the hostile conditions during the last glacial maximum. Apart from some purported cases of interaction between species (e.g. Nasri *et al.*, 2008) this establishment is largely considered to have been based on the individual responses of species (Davis, 1976; Huntley & Birks, 1983; Huntley & Webb, 1989). The rates and degree of occupation of available habitat by different species is related to the number and location of glacial refugia, the individual fitness of species and to life history traits, including the mechanism of seed dispersal.

In southeastern Australia cool temperate rainforests are mostly comprised of two tree species, *Nothofagus cunninghamii* (Nothofagaceae) and *Atherosperma moschatum* (Atherospermataceae), with largely overlapping distributions but distinct dispersal mechanisms. *Atherosperma moschatum* has plumose wind dispersed seeds that are considered to allow the dispersal of this species over long distances (Hickey *et al.*, 1982; Neyland & Brown, 1993; see Fig. 1). On the other hand, *N. cunninghamii* has gravity dispersed seed, and chloroplast phylogeographic evidence indicates that the recovery of this species after the height of last glacial conditions, the last glacial maximum (LGM) ~ 18,000 years ago, involved limited migration from glacial refugia that occurred within multiple parts of its current range (Chapter 2). Similar inferences were made for the fleshy-fruited shrub *Tasmannia lanceolata* (Winteraceae) (Chapter 3), a plant common in the understorey of cool temperate rainforest that also has a similar distribution to *A. moschatum*.

Fig. 1 Plumose achene of *Atherosperma moschatum* (centre, trapped in spider's web).

Some evidence suggests that *A. moschatum* had a divergent response to these species, with the species widespread distribution hypothesised to be a result of its presumably mobile wind dispersed seeds (Read & Busby, 1990). For example, the occurrence of the species in eastern Gippsland of Victoria (see Fig. 2), where gravity dispersed *N. cunninghamii* is absent, has been considered a result of the greater ability of *A. moschatum* to cross dry corridors by long-distance dispersal (Howard & Ashton, 1973). A similar explanation has been made for the presence of *A. moschatum* in small, topographically protected sites in the predominantly dry east of Tasmania, locations where *N. cunninghamii* is also absent (Gilbert, 1959; Neyland & Brown, 1993).

However, little evidence is currently available to support the hypothesis that recolonisation during the Holocene of *A. moschatum* has been geographically extensive or as to the locations of glacial survival of this species. Firstly, an isozyme study of *A. moschatum* by Shapcott (1994) observed no patterns of isozyme variation consistent with establishment of current populations in Tasmania via either recent dispersal or glacial refugia. Secondly, the fossil record of *A. moschatum* is uninformative as to the location of this species during the LGM due to the rarity of

LGM pollen or macrofossils of rainforest species in general and the poor representation of *A. moschatum* pollen in sediments (Ladd, 1979a; Macphail, 1984; Hopf *et al.*, 2000). Pollen, albeit only one or two pollen grains, of the species has been found in LGM sediments in western Tasmania (Macphail & Colhoun, 1985; Colhoun *et al.*, 1999; Hopf *et al.*, 2000). On mainland Australia trace quantities of *A. moschatum* pollen have been found ~ 32,000 years before present in the Australian Alps (Kershaw *et al.*, 2007), however no LGM pollen record has been found, including sites from the Central Highlands of Victoria containing LGM pollen of *N. cunninghamii* (McKenzie, 1997). The distinctiveness of the northernmost populations of *A. moschatum*, however, in the Blue Mountains and Barrington Tops region (Fig. 2) in morphology (Schodde, 1969) and at the isozyme level suggest long term isolation of these populations (Floyd, 1990; Shapcott, 1994). Populations of the species in these two areas have been classified as a separate subspecies, subsp. *integrifolium* (Schodde, 1969; Foreman & Whiffen, 2007).

This study aims to improve our understanding of the response of *A. moschatum* to past climatic perturbations, including the location of glacial refugia and the importance of wind dispersal by seed, by investigating the chloroplast DNA (cpDNA) phylogeography of *A. moschatum*. Specifically this study has two aims:

1. Identify chloroplast variation across the range of *Atherosperma moschatum*.

2. Assess whether the patterns of chloroplast DNA variation is consistent with expectations of widespread colonization of the species during the Holocene or with a history of limited expansion from multiple refugia.

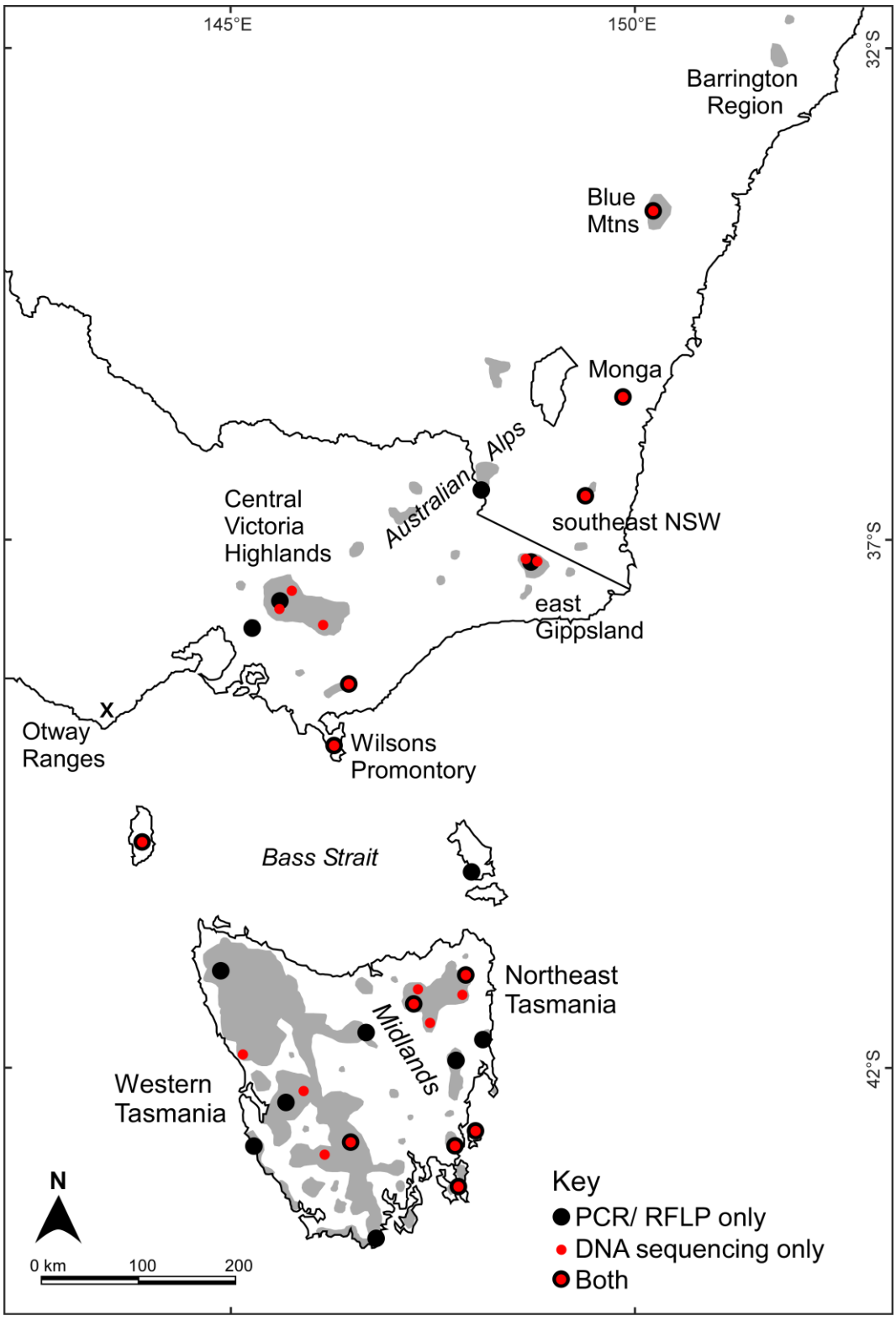


Fig. 2 Natural distribution of *Atherosperma moschatum* (grey shading). The circles indicate the locations of samples used for screening for chloroplast variation using PCR-RFLP only (black circles), DNA sequencing only (red small circles) or both (red and black circles). The X indicates the possible pre-European occurrence of the species in the Otway Ranges where it is now absent (Lunt, 1992).

Materials and Methods

The study species

Atherosperma moschatum Labill. (southern sassafras or black sassafras) is the only species within the genus. The species is the geographically most widespread cool temperate rainforest tree in southeastern Australia, extending across 12 degrees of latitude (Read & Hill, 1988a) from southern Tasmania to the headwaters of the Tia River north of Barrington Tops, New South Wales (NSW) (Floyd, 1989; Fig. 2). This evergreen tree species can reach 45 m in height (Curtis & Morris, 1994) and is usually dioecious, although both sexes can occasionally be found on the same tree (Jones, 1982). In Tasmania, *A. moschatum* occurs from sea-level to ~ 1100 metres above sea-level (masl) (Read & Hill, 1988a) where it can occur as a shrub, although unhealthy plants are sometimes observed at higher altitudes (G. Jordan pers. comm.). On the mainland, the species is confined to higher altitude forests, above 350 masl in Victoria, 700 – 1000 masl in southeastern NSW, and generally above 1200 masl in the most northern part of its range in the Barrington Tops region (Floyd, 1990), where it has a very localized occurrence (Read & Hill, 1989).

Across its latitudinal range, *A. moschatum* co-occurs with the three major cool temperate rainforest trees in southeastern Australia; *Nothofagus cunninghamii* in Tasmania and Victoria, *(*Howard, 1973); *Eucryphia moorei* in the Monga National Park west of Batemans Bay, NSW (Floyd, 1990; Mackey *et al.*, 1999) and *Nothofagus moorei* in the Barrington Tops region of NSW (Fraser & Vickery, 1938). However, *A. moschatum* also occurs in other forest types, including forest dominated by *Ceratopetalum apetalum* in the Blue Mountains of NSW (Lundie-Jenkins, 1993; Selkirk *et al.*, 2001), and *Elaeocarpus holopetalus* in eastern Gippsland and parts of southeastern NSW (Forbes *et al.*, 1982; Floyd, 1990; Cameron, 1991). In addition, the species is also the sole cool temperate rainforest tree in parts of eastern Tasmania (Kirkpatrick, 1981; Neyland & Brown, 1993), in the Dandenong Range of Victoria (Howard & Ashton, 1973) and localised parts of the Australian Alps (Gellie, 2005). In Tasmania and Victoria *A. moschatum* also extends into the wet *Eucalyptus* forest sub-canopy (Gilbert, 1959; Ashton, 2000).

Sampling

Leaves were sampled from 142 *A. moschatum* individuals from most parts of the species' range in Tasmania and mainland Australia. Samples were collected a minimum of ~ 1 km apart except for some isolated populations where at least 2 samples were taken where possible. Three samples of *A. moschatum* subsp. *integrifolium* were sampled from two locations in the Blue Mountains, NSW. In addition, three samples of *A. moschatum* from Penance Grove, Monga NP (NSW) were included. This population was not included either in the isozyme survey by (Shapcott, 1994) and in the classification of the subspecies *A. moschatum* subsp. *integrifolium* (Schodde, 1969; Foreman & Whiffen, 2007). Samples from Monga NP match the description of *A. moschatum* subsp. *integrifolium* based on the entire margin or rare toothing of the adult leaves, and strong pubescence of the stems (specimen examined: A. M. Buchanan 13972 (HO 312807)), although the trees at Monga NP can be considerably taller (up to 30 m tall) (Floyd, 1990), than the 10 m maximum height described for the subspecies *integrifolium* (Foreman & Whiffen, 2007). Information on the distribution of *A. moschatum* was obtained from the Natural Values Atlas (Department of Primary Industries and Water, Tasmanian Government (http://www.naturalvaluesatlas.dpiw.tas.gov.au)) for Tasmania, and Australia's Virtual Herbarium (http://www.anbg.gov.au/avh/cgi-bin/avh.cgi), Floyd (1989, 1990), Coyne (2001) and Ashton (2000) for mainland populations. For use as outgroups, one sample each of three of the six other genera of Atherospermataceae, were sampled. These were the Chilean endemic *Laurelia sempervirens* (grown in a private collection by Ken Gillanders, southern Tasmania), *Nemuaron vieillardii*, collected from Mont Dzumac, New Caledonia, and *Daphnandra tenuipes* from the Border Ranges National Park, northeast NSW.

Molecular methods

Total genomic DNA was extracted from 0.25 g of adult leaves using the Qiagen DNeasy Plant Mini Kit (QIAGEN Pty Ltd Vic, Australia). DNA quantity and quality were assessed by agarose gel electrophoresis with ethidium bromide staining and comparison with a standard molecular weight marker (Lambda *Hind*III).

PCR-RFLP and DNA sequencing were used to discover cpDNA variation in *A. moschatum*. For the preliminary survey using PCR-RFLP, a total of 15 pairs of universal chloroplast primers (Table 1) were tested for success of amplification in *A.*

moschatum. All PCR reactions were performed in a total volume of 25 µl containing 2.5 mM $MgCl_2$; 100 µg/mL of Bovine Serum Albumin; 80 µM each of dATP, dCTP, dGTP and dTTP; 5 pM of each primer; 1 x PCR buffer (67 mM Tris-HCl, 16.6 mM $(NH_4)_2 SO_4$, 0.5% Triton X-100 and 5 µg of gelatin); two units of *Taq* DNA polymerase; and approximately 10 ng of genomic DNA (1-2 uL of DNA). PCR amplification was performed with a MJ Research PTC-225 Tetrad thermocycler (GMI, Inc. Minn., USA) as follows: an initial melt of 4 min at 94°C; 30 cycles of 45 sec at 92°C, 45 sec at annealing temperature (see Table 1 for details of annealing temperatures of each fragment), 4 min at 72°C; and a final extension for 10 min at 72°C. PCR products that were successful in amplification of single banded and high yielding products were subsequently used for preliminary screening of 24 samples by PCR-RFLP for *A. moschatum* (Fig. 2). These samples were chosen to represent the whole range of the species with particular emphasis on isolated populations of the species in eastern Tasmania and mainland Australia.

Table 1 The chloroplast primer pairs, including annealing temperature, tested in *Atherosperma moschatum* for screening for chloroplast variation using PCR-RFLP.

Name	Primer Forward (5'–3')	Primer Reverse (3'–5')	Annealing temperature (Ta°C)	Reference
AE	petAf	psbEr	42	a
L106	rbcLf	orf106	42	b
pB	clpp	psbB	50	b
HI	atpH	atpI	50	b
OA	orf184	petA	52	b
B2B3	psbB	petB	52	b
DT	trnD	trnT	54	c
BD	petB	petD	54	b
SG	trnS	trnG	54	d
K2Q	trnK	trnQ	57	e
AS	psaA	trnS	57	c
CS	psbC	trnS	58	c
ST	trnS	trnT	58	c
HK	trnH	trnK	62	c
Sf_M	trnS	$trnf_M$	62	c

a, Fofona *et al.* (1997); b, Grivet *et al.* (2001); c, Demesure *et al.* (1995); d, Hamilton (1999); e, Dumolin-Lapegue *et al.* (1997b).

All successful PCR products were digested with the restriction endonucleases *Taq*I, *Hinf*I and *Hae*III following the instructions of the manufacturer. An additional restriction endonuclease, *Hpy*188III, was used for *A. moschatum* fragments BD, SG and Sf_M. All digestions were undertaken in a total reaction volume of 20 µl containing 5-10 µL of PCR product. The products of the restriction digests were size fractionated in 2.2% agarose gel with TBE buffer at 100 volts for 90-150 mins. Restriction fragment length polymorphisms (RFLPs) were identified visually by comparing restriction fragment patterns between samples.

For DNA sequencing, a total of eight different universal chloroplast primer pairs were tested for success of amplification in *A. moschatum* (Table 2). All primers pairs that were successful in amplification of single-band products were used to amplify a subset of 24 samples in *A. moschatum* (a different subset to that used for PCR-RFLP; see Fig 2).

Table 2 Primer pairs, including annealing temperatures, tested in *Atherosperma moschatum* for screening for chloroplast variation using DNA sequencing.

Fragment	Primer forward (5'–3')	Primer reverse (3'–5')	Annealing temperature (Ta°C)	Reference
*atp*B-*rbc*L	atpB-1	rbcL-1	55	a
K1-*mat*K1	K1	matK1	51	b
*mat*K6-K2	matK6	K2	51	b
*pet*N1-*psb*M	petN1	psbM2R	51	c
*psb*M-*trn*D	psbM2	trnD	51	c
*trn*T-*trn*L	a	b	51	e
*trn*L intron	c	d	50	e
*trn*L-*trn*F	e	f	50	e

a, Chiang *et al.* (1998); b, Grivet & Petit (2002); c, Lee & Wen (2004); d, Shaw *et al*, (2007); e, Taberlet *et al.* (1991).

PCR conditions were as follows: for *atp*B-1-*rbc*L-1, an initial 4 min at 94°C, followed by 35 cycles of 45 sec at 94°C, 1 min and 15 secs at 55°C and an extension for 1 min and 15 secs at 72°C, and a final extension step for 10 min at 72°C; for K1-*mat*K1 and *mat*K6-K2, an initial 4 min at 94°C, followed by 35 cycles of 45 sec at 94°C, 1 min at 51°C and extension for 1.5 min at 72°C, and a final extension step for 10 min at 72°C;

for *psb*M2-*trn*D, *pet*N1-*psb*M2R and a-b reactions, an initial 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 2 mins at 51°C and extension for 2 min at 72°C, and a final extension step for 10 min at 72°C; for e-f and c-d, an initial 1 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 50°C and extension for 45 secs at 72°C, and a final extension step for 7min at 72°C. PCR quality was assessed by gel electrophoresis on 1.2% agarose gels and staining with ethidium bromide. In preparation for DNA sequencing, PCR products were purified using the Qia-Quick PCR purification kit (QIAGEN Pty Ltd Vic, Australia). The primer used to sequence each fragment is shown in Table 3. In most cases the forward primer was used.

Table 3 The primer(s) used to sequence each fragment for *A. moschatum*.

Fragment	*A. moschatum*
atpB-rbcL	both
K1-matK1	K1
matK6-K2	matK6
petN1-psbM	both
psbM-trnD	psbM2
trnT-trnL	a
trnL intron	c

Sequencing reactions were performed in a MJ Research PTC-225 Thermal Cycler using ABI Prism Bigdye Terminator v 3.0 Cycle Sequencing Kits (Applied Biosystems, CA, USA) with AmpliTaq DNA polymerase (Applied Biosystems, CA, USA) and fragment separation was done on a 3730*xl* DNA Analyzer (Applied Biosystems, CA, USA). Sequences were aligned using Sequencher 4.5 (Gene Codes Corporation, MI, USA), and checked by eye for incorrect base calls, single nucleotide polymorphisms (SNPs), DNA insertions and deletions (indels) and simple sequence repeat (SSR) variations. DNA polymorphisms detected in only one sample were checked by repeating the PCR and the sequencing reaction. Fragments that contained cpDNA variation in the subset of 24 samples were sequenced for the remaining 128 samples.

DNA sequence and haplotype analysis

The nucleotide diversity (π) (Nei, 1987) of the cpDNA sequences were calculated separately for each chloroplast fragment with the program DnaSP version 5.00.02

(Rozas *et al.*, 2003). In order to do this analysis the two sequence lengths obtained for the *pet*N–*psb*M intergenic spacer were concatenated into a single alignment. For comparison of nucleotide diversity, this analysis was also undertaken for the sequence data of the widespread cool temperate rainforest plants *N. cunninghamii* (Chapter 2) and *T. lanceolata* (Chapter 3). For the analysis of nucleotide diversity two separate analyses (populations) were completed for each species using the whole sampled range of each species and using samples only from where the distribution of the three species overlap. In addition, tests of neutrality, Tajima's *D* (Tajima, 1989) and Fu and Li's *D** and *F** (Fu & Li, 1993), were conducted for *A. moschatum* using DnaSP version 5.00.02. For all *A. moschatum* haplotypes a median-joining network, with equal weighting of all characters, was constructed using *Network 4.5.0.2* (Bandelt *et al.*, 1999). The spatial structuring of *A. moschatum* haplotypes was investigated using the single nearest geographic neighbour for each sample that was determined using a specially written macro in SAS 9.1 (SAS Institute Inc., Cary, NC, USA). This program also performed a permutation test (Manly, 1997) with 10,000 randomised repeats testing whether the nearest neighbour of each sample was more often of the same haplotype than expected by chance (a proxy of spatial structure). This analysis was undertaken separately for all samples, mainland Australian samples, Tasmania, and also for eastern and western Tasmania, with western Tasmania defined as any samples occurring west of a line passing through the northern and southern midlands of the island.

Phylogenetic analysis

Phylogenetic analysis of the aligned sequences was undertaken using exhaustive searches for most parsimonious trees, using the program PAUP* version 4.0b10 (Swofford, 2000). All characters, including indels and SSR regions, were treated as unordered and of equal weight. Indels were scored as binary characters, while SSR regions were scored as multistate characters with a maximum of 4 states. Eight bases of the *trn*T-*trn*L intron were excluded from the analysis because alignment of this region was ambiguous. Branch support was assessed by bootstrap analysis (Felsenstein, 1985) with 1000 bootstrap replicates using the same search parameters as those in the parsimony analysis. *Laurelia sempervirens*, *Nemuaron vieillardii*, and *Daphnandra tenuipes* were used as outgroups. The K1-matK1 fragment and petN-psbM intergenic spacer sequences were of poor quality for *D. tenuipes* and were scored as missing data. Initially, most parsimonious trees were generated

independently for each chloroplast fragment in order to assess whether conflict existed between these data sets (i.e. non-congruent clades with greater than 50% bootstrap support) prior to the concatenation of the five data sets into a single alignment as described by Renner *et al.* (2000).

Results

PCR-RFLP

In total nine primer pairs of the 15 tried for *A. moschatum* produced single banded and high yielding fragments that were suitable for PCR-RFLP analysis (totalling 15,400 bp; Table 4). PCR-RFLP failed to detect any cpDNA variation among the subset of 24 samples after screening with 30 fragment/ restriction endonuclease combinations (Table 5).

Table 4 PCR primer pairs that were successful in amplifying *A. moschatum* cpDNA indicated by the approximate sizes in base pairs of the fragments. The dashes indicate fragments that were not amplified or otherwise not suitable for PCR-RFLP.

Fragment	*A. moschatum*
AE	2500
L106	–
pB	2800
HI	1000
OA	–
B2B3	–
DT	1300
BD	1500
SG	1000
K2Q	–
AS	–
CS	–
ST	1400
HK	2500
Sf_M	1400

Table 5 The number of restriction sites after digestion by four restriction endonucleases of the subset of 24 samples of *Atherosperma moschatum*. The dashes indicate fragment/ restriction endonuclease combinations that were not undertaken.

Fragment	*Taq*I	*Hinf*I	*Hae*III	*HpY188*III
AE	3	3	1	–
pB	4	6	3	–
HI	1	2	0	–
DT	3	2	1	–
BD	3	4	1	4
SG	2	3	1	2
ST	0	2	1	–
HK	2	3	3	–
Sf_M	3	3	0	2

DNA Sequencing

Of the eight primer pairs tested all produced a single banded product suitable for DNA sequencing, except for the *trn*L-*trn*F fragment (Table 6) which did not amplify, a finding also observed by Renner *et al.* (2000) for *A. moschatum*.

Table 6 Primer pairs that were successfully amplified in *Atherosperma moschatum* for DNA sequencing are indicated by the approximate sizes in base pairs of the fragments. The dash indicates the failure to amplify the *trn*L-*trn*F fragment.

Fragment	Size (bp)
*atp*B-*rbc*L	950
K1-*mat*K1	950
*mat*K6-K2	700
*pet*N1-*psb*M	1300
*psb*M-*trn*D	1300
*trn*T-*trn*L	850
*trn*L intron	650
*trn*L-*trn*F	–

In the preliminary screening for cpDNA variation using 24 samples at least one DNA sequence polymorphism was observed in the K1–*mat*K1, *mat*K6–K2, *pet*N1–*psb*M, *trn*T–*trn*L and the *trn*L intron fragments. No variation was observed for the *psb*M-*trn*D and *atp*B-*rbc*L fragments. In the subsequent screening of all remaining samples (128 samples) no new sequence polymorphisms were detected. Aligned sequences

lengths and GenBank accession numbers for each fragment used in the preliminary screening and full sample set are shown in Table 7 (see Appendix 2 for GenBank accession numbers for outgroups). Overall a total of five SNPs (2 transitions and 3 transversions) and three one bp indels (Table 8) were observed within the 3,295 bp of aligned sequence obtained for all 142 *A. moschatum* samples. All one bp indels were associated with AT pure SSRs over 10 bp in length. Two other SSR regions of 10 bp or over in length were not variable across the 142 samples.

Table 7 The aligned length obtained for cpDNA fragments sequenced in *A. moschatum* and their GenBank accession numbers. Size differences for fragments are due to SSR length variations. The fragments in bold did not display chloroplast variation within the subset of 24 of *A. moschatum* samples.

Fragment	Aligned length (bp)	GenBank accession numbers
***atp*B–*rbc*L F**	**398**	**FJ861006**
***atp*B–*rbc*L R**	**297**	**FJ861007**
K1–*mat*K1	615-616	FJ860982–FJ860987
*mat*K6–K2	779	FJ860988–FJ860993
*pet*N1–*psb*M F	188-189	FJ860994–FJ860999
*pet*N1–*psb*M R	678	FJ861000–FJ861005
***psb*M–*trn*D**	**652**	**FJ861008**
*trn*T–*trn*L	562-563	FJ860970–FJ860975
*trn*L intron	470	FJ860976–FJ860981

Table 8 Single nucleotide polymorphisms (characters 2, 4, 5, 6 and 8) and simple sequence repeat (SSR) polymorphisms (characters 1, 3 and 7) defining the six chloroplast DNA haplotypes observed in *Atherosperma moschatum*, shown in comparison to the most frequent haplotype H1. The dot indicates congruence with the state in haplotype H1. The states at variable sites in *A. moschatum* are also shown for outgroups, *Laurelia sempervirens, Nemuaron vieillardii* and *Daphnandra tenuipes*. These outgroups varied at 137 other sites including 123 SNPs and 14 length variations.

Haplotype	*trn*T-*trn*L 1	*trn*L intron 2	K1-*mat*K1 3	K1-*mat*K1 4	K1-*mat*K1 5	*mat*K6-K2 6	*pet*N-*psb*M f 7	*pet*N-*psb*M r 8
H1	A(11)	T	T(10)	G	C	G	A(12)	A
H2	.	.	.	.	T	.	.	.
H3	.	.	.	.	.	.	A(11)	.
H4	.	.	.	.	.	A	.	.
H5	A(10)	A	T(11)	.	.	.	.	.
H6	.	.		T	.	.	.	C
L. sempervirens	A(13)	.	T(11)	.	T	.	A(13)	.
N. vieillardii	.	.	T(11)	.	.	.	A(10)	.
D. tenuipes	A(9)	.	?	?	?	.	?	?

Phylogenetic relationships and distribution of haplotypes

Overall 2,818 base pairs of aligned sequences were obtained for *A. moschatum* and all outgroups, except *D. tenuipes*. The alignment contained 112 substitutions, 10 indels and 7 SSR regions of which 36 characters were parsimony informative. Most of the divergence was in the K1-*mat*K1, *mat*K6-K2 and *pet*N-*psb*M fragments. Parsimony analysis resulted in 4 trees (length = 142; consistency index = 0.99; retention index = 0.97) (Fig. 3). Bootstrap support is high for the monophyly of *A. moschatum* haplotypes (BP = 100%; Fig. 3) and for a clade containing *L. sempervirens* and *N. vieillardii* (BP= 97%). The median joining network (Fig. 4) is consistent with the phylogeny.

The eight polymorphisms observed in *A. moschatum* defined 6 haplotypes (Table 8). Three haplotypes were observed among the 107 samples from Tasmania. Haplotype H1 (61% of all samples) was widespread across Tasmania including the Bass Strait islands, but was absent from mainland Australia (Fig. 5). This haplotype is inferred as being ancestral (Fig 4) based on the combined evidence of the wide geographical distribution of this haplotype (Templeton, 1998), and the interior status of the

haplotype in the network (Cruzan & Templeton, 2000), that is, all other haplotypes observed in *A. moschatum* are most parsimoniously derived from it. Haplotype H2, differing by one SNP, was rare (2.8% of all samples) and was observed in northeast Tasmania (2 samples), north-central Tasmania (Liffey Falls; 1 sample) and south-eastern Tasmania (Tasman Peninsula; 1 sample). Haplotype H4 (11.3%), differing by one SNP in the *mat*K6-K2 intron, was observed only in central and southwest Tasmania, Bruny Island and the Tasman Peninsula. This latter site contained all three Tasmanian haplotypes (Fig. 5), and is thus the most diverse region in Tasmania. Haplotype H3 (20.5%) was the only haplotype observed in all Victorian populations and in southern NSW at Brown Mountain and the Geehi area, Kosciusko National Park (Fig. 5). This haplotype differed from H1 by a single SSR length variant in the *pet*N–*psb*M intergenic spacer. Both of the most northern populations sampled were found to harbour a unique haplotypes. All three samples from the isolated population at Monga National Park had a haplotype H5 (2.2% of samples) differing from H1 by two SSRs and one SNP (Fig. 4). The Blue Mountains samples, from Leura Falls (1 sample) and Bonnie Doon Falls (2 samples) located approximately 3.3 km apart harboured haplotype H6 (2.2%), differing from H1 by two SNPs (Fig.4).

Analyses of nearest geographic neighbours showed that individuals were more likely ($P < 0.001$) to be geographically nearest to individuals of the same haplotype (105 of 142) than to individuals of different haplotypes (Table 9). However, this analysis was strongly impacted by the low diversity of haplotypes observed in the species. In western Tasmania, where two haplotypes were observed the level of spatial structuring was not significant.

Table 9 Nearest neighbour analysis of western Tasmanian, Tasmanian and mainland Australian samples of *Atherosperma moschatum*. The number of observed individuals (*n*) with a nearest neighbour of the same haplotype is shown with probabilities.

	P	observed	expected
Western Tasmania	0.019	63	57.2
Tasmania	0.003	83	76
Mainland Australia	0.005	28	24.3
All samples	< 0.001	105	62.9

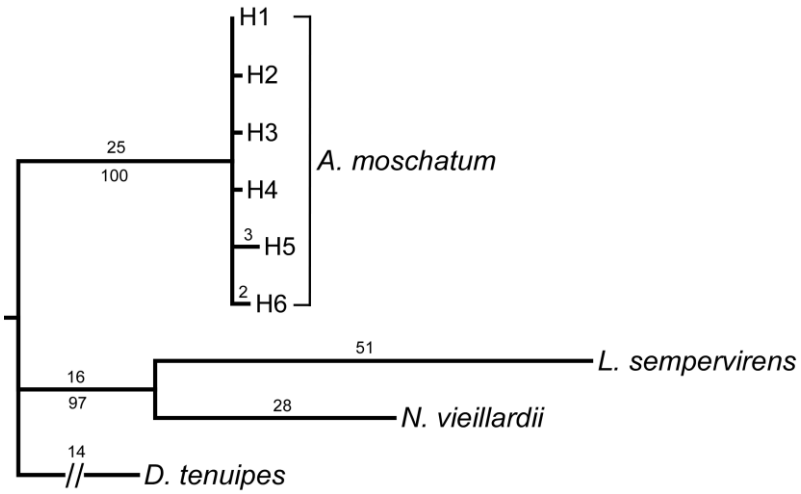


Fig. 3 One of four most parsimonious trees obtained from chloroplast sequence characters in *Atherosperma moschatum* and outgroups. Branch lengths (the inferred number of single base pair substitutions and indels on a branch) of greater than one are indicated above branches while bootstrap values above 50% are shown above branches. The broken line indicates that data was missing for *D. tenuipes*.

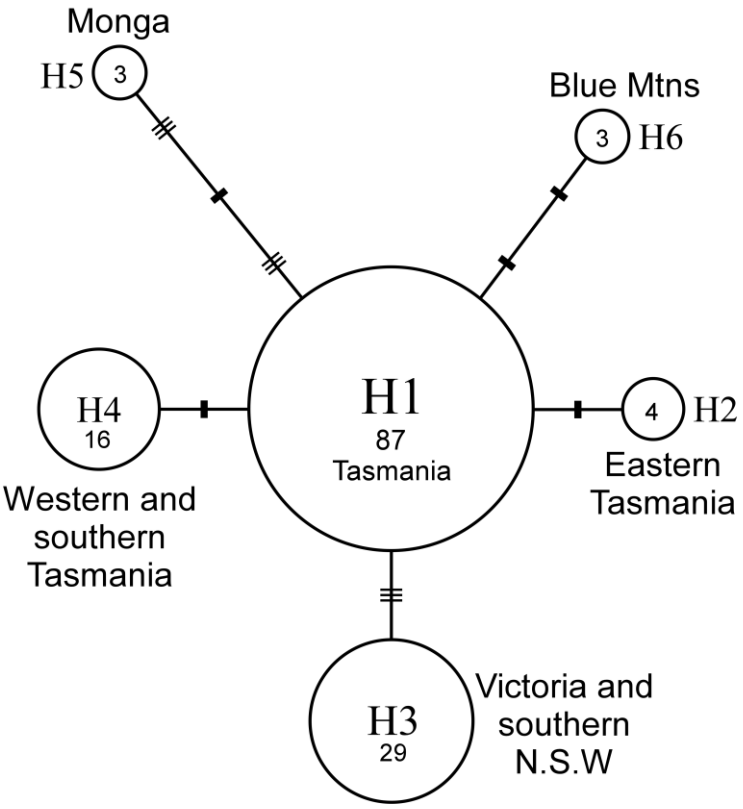


Fig. 4 Median-joining network of the six haplotypes observed in *Atherosperma moschatum*. The area of the circles is proportional to the frequency of each haplotype. The number of samples of each haplotype is indicated inside each circle. Lengths of lines connecting each haplotype are proportional to the number of character differences between them. Single nucleotide polymorphisms are indicated by solid bars and simple sequence repeat (SSR) length variations indicated by three stacked lines.

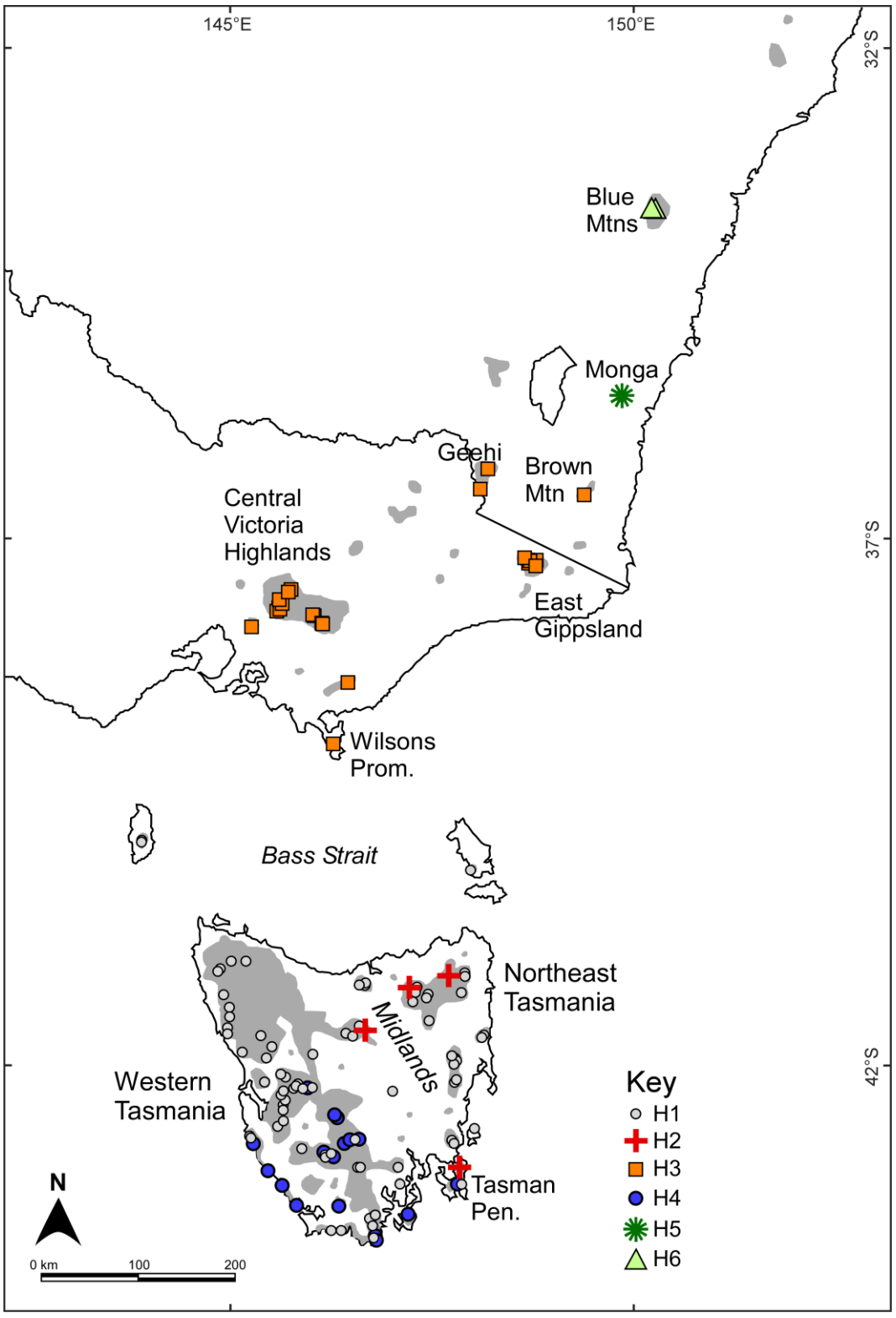


Fig. 5 Distribution of haplotypes observed in *Atherosperma moschatum*. Grey areas indicate the natural distribution of the species.

Nucleotide diversity and tests for selection

The mean nucleotide diversity for all 142 samples of *A. moschatum* was 0.00011, 8.6 and 5.9 times lower than that observed in *N. cunninghamii* (0.00098) and *T. lanceolata* (0.00068), respectively (Table 10). These differences increased in magnitude when nucleotide diversity was calculated from samples only from where the distribution of all three species overlap (Table 11), with the nucleotide diversity of *A. moschatum* 11.5 times lower than *N. cunninghamii*, the increase largely due to the exclusion of the two northern populations of *A. moschatum*. The Tajima's *D* and Fu and Li's *D** and *F** statistics neutrality test were all insignificant, indicating there is no reason to believe that the cpDNA of *A. moschatum* was under selection.

Table 10 The nucleotide diversity (π) estimated for *N. cunninghamii*, *T. lanceolata* and *A. moschatum*, shown for each chloroplast fragment. The highest values for each fragment and highest mean values and are indicated in bold.

	N. cunninghamii		*T. lanceolata*		*A. moschatum*	
	π[a]	SD	π	SD	π	SD
*trn*S–*trn*f$_M$	0.00216	0.00014	–	–	–	–
*rps*16 intron	0.00005	0.00004	–	–	–	–
*psb*M–*trn*D	0.00093	0.00018	**0.00243**	0.00021	–	–
*trn*L–*trn*F	**0.00162**	0.00026	0.00035	0.00011	–	–
*pet*N1–*psb*M	0.00014	0.00005	**0.00027**	0.00008	0.00005	0.00003
K1–*mat*K1	–	–	**0.00044**	0.00007	0.00016	0.00006
*mat*K6–K2	–	–	**0.00038**	0.00005	0.00026	0.00005
*trn*L intron	–	–	**0.00018**	0.00005	0.00009	0.00005
*trn*T–*trn*L	–	–	–	–	0.00000	0.00000
Mean	**0.00098**		0.00068		0.00011	

[a] The average number of nucleotide differences per site between any two randomly chosen DNA sequences.

Table 11 The nucleotide diversity (π) estimated for *N. cunninghamii*, *T. lanceolata* and *A. moschatum* calculated only from samples where the distribution of all three species overlap. The highest values for each fragment and highest mean value are indicated in bold.

	N. cunninghamii		*T. lanceolata*		*A. moschatum*	
	π	SD	π	SD	π	SD
*trn*S–*trn*f_M	0.00220	0.00014	–	–	–	–
*rps*16 intron	0.00005	0.00004	–	–	–	–
*psb*M–*trn*D	0.00109	0.00018	**0.00191**	0.00025	–	–
*trn*L–*trn*F	**0.00166**	0.00026	0.00007	0.00005	–	–
*pet*N1–*psb*M	0.00014	0.00006	**0.00032**	0.00010	0.00000	0.00000
K1–*mat*K1	–	–	**0.00024**	0.00006	0.00012	0.00005
*mat*K6–K2	–	–	**0.00044**	0.00006	0.00032	0.00006
*trn*L intron	–	–	**0.00021**	0.00007	0.00000	0.00000
*trn*T–*trn*L	–	–	–	–	0.00000	0.00000
Mean	**0.001028**		0.000532		0.000088	

Discussion

Low chloroplast variation

The low chloroplast variation observed in *A. moschatum* appears to be unusual for chloroplast phylogeographic studies that are based on the sequencing of multiple regions of the chloroplast. Low chloroplast variation has been observed in a number of studies principally using the PCR-RFLP technique, for example, in *Aucoumea klaineana* (Muloko-Ntoutoume *et al.*, 2000), *Corylus avellana* (Palme & Vendramin, 2002), *Erinus alpinus* (Stehlik *et al.*, 2002) and *Nothofagus antarctica* (Pastorino *et al.*, 2008). Although direct comparisons of cpDNA diversity with other plant species are difficult, due to the fact that different combinations of cpDNA fragments have been used to discover variation, the nucleotide diversity of *A. moschatum* was lower than 12 other plant species where nucleotide diversity has been calculated (Table 12).

Table 12 Chloroplast nucleotide diversity (π) (Nei, 1987) for 12 plant species in comparison to *A. moschatum* (bottom of table).

Species	Lifeform	Region (*N*)[a]	Total bp[b]	π	Reference
Fagopyrum leptopodum	annual herb	China (10)	2530	0.00195	1
Cunninghamia konishii	Tree	Taiwan (64)	1888	0.00190	2
Fagopyrum statice	annual herb	China (5)	2498	0.00144	1
Nothofagus cunninghamii	Tree	SE Australia (213)	2164	0.00098	This study
Castanopsis carlesii	Tree	Taiwan (201)	1663	0.00095	3
Corylis avellana	Tree	Europe (7)	2468	0.00093	4
Lagarostrobos franklinii	Tree	W Tasmania (96)	892	0.00093	5
Castanopsis glauca	Tree	Taiwan (140)	1980	0.00074	6
Tasmannia lanceolata	Shrub	SE Australia (244)	3190	0.00068	This study
Trochodendron aralioides	Tree	Taiwan (80)	1102	0.00052	7
Machilus kusanoi	Tree	Taiwan (106)	1041	0.00051	8
Machilus thunbergii	Tree	Taiwan (110)	1031	0.00031	8
Atherosperma moschatum	Tree	SE Australia (142)	3295	0.00011	This study

a, the region from where samples of each species were obtained, with the number of samples investigated per species shown within the brackets. b, concatenated sequence length (bp), including gaps, obtained per species.

1, Ohsako & Ohnishi (2001), 2, Hwang *et al.* (2003), 3, Cheng *et al.* (2005), 4, Palme & Vendramin (2002), 5, Clark & Carbone (2008), 6, Huang *et al.* (2002), 7, Huang *et al*. (2004), 8, Wu *et al.* (2006).

Towards an explanation of low diversity in A. moschatum

The low diversity of *A. moschatum* is of some interest as *Atherosperma* is purported to be a genus with a long history since molecular dating have provided an age of 69.5 my for *Atherosperma*, an estimate based on a phylogeny including all genera in the family and the sequencing of six chloroplast regions (comprising 4,203 bp, excluding gaps) (Renner, 2004) and a minimum age for the family of 88 my (Mohr, 1998). A single leaf fossil that is indistinguishable from the extant species provides evidence for the occurrence of *A. moschatum* in the Early Pleistocene (~1 my ago) of western Tasmania (Hill & Macphail, 1985). However, it is plausible that the species is much older given that the leaves are unlikely to be fossilised due to their rapid decay (Howard, 1973b; Carpenter & Horwitz, 1988).

One hypothesis to explain the low variation observed in *A. moschatum* is that mutation rates may be low in the genus. Rates of chloroplast substitution can vary considerably in plants (Bousquet *et al.*, 1992), for example, as a result of differences in DNA repair mechanism (Britten, 1986). Different rates of mutation have been inferred to explain differences in haplotype diversity between species with similar

ranges. For example, Palme *et al.* (2003b) observed high haplotype diversity across the range of *Salix caprea* when compared to similar studies in ten other tree species and attributed this greater diversity to a higher mutation rate in *Salix*. There is no clear evidence for slower chloroplast evolution in *A. moschatum* compared to other plant species. The cpDNA substitution rate in the family Atherospermataceae has been calculated as between 1.4 to 2.4 x 10^{-4} substitutions per site per million years (SSMY) for *rbcL* (Renner *et al.*, 2000). This is comparable to the rates estimated for Winteraceae (0.98 to 1.47 x 10^{-4} SSMY) and Nothofagaceae (2.10 x 10^{-4} SSMY) (Albert *et al.*, 1994), the two families containing *T. lanceolata* and *N. cunninghamii*. However, there is considerable uncertainty in these estimates because substitution rates are calculated in various ways (Renner *et al.*, 2000), the accuracy of calibration points is crucial in estimates (Kay *et al.*, 2006) and rates of evolution of *rbcL* may differ from other chloroplast regions.

Another hypothesis for the low variation observed in *A. moschatum*, in contrast to that in the co-occurring *N. cunninghamii* and *T. lanceolata,* is that *A. moschatum* may have had greater reductions in population size during the past. *Atherosperma moschatum* has marked ecological and physiological differences compared to *N. cunninghamii* and *T. lanceolata*, being rarer at high altitudes and usually occurring in the ameliorated conditions of the cool temperate rainforest canopy (Hill *et al.*, 1988; Read, 1999), a position it is able to maintain through its ability to regenerate continuously in closed forest (Read & Hill, 1988b) due to its greater shade tolerance and through asexual reproduction (Read, 1985). Physiological experiments have confirmed *A. moschatum* as being the least cold tolerant of all the tree species in cool temperate rainforest (Read & Hill, 1989; Read & Busby, 1990; Feild & Brodribb, 2001). In contrast *N. cunninghamii* has a wide tolerance to temperature occurring in shrub form in the subalpine zone, while *T. lanceolata* is common in the alpine zone and appears to have been common in open vegetation in some parts of western Tasmania during the last glacial. During the LGM, treeless conditions within currently forested parts of eastern Australia are known to have extended as far north as Barrington Tops (Sweller & Martin, 2001). Stands of tall forest (i.e. like present day) would not have been extensive due to the lowering of the tree-line almost to current sea-level (Colhoun, 1985a). Therefore, the habitat for *A. moschatum* may have been considerably more restricted than those of *N. cunninghamii* and *T. lanceolata* during the cold and dry conditions of the glacials. This would result in very small

populations of *A. moschatum*, purging existing variation (i.e. causing bottlenecks). Such bottlenecks may have occurred repeatedly over the Pleistocene glacial/interglacial cycles. The long-branch length leading to the observed haplotypes in *A. moschatum* supports the presence of genetic bottlenecking within the species. The higher diversity of haplotypes in Tasmania than the majority of the species range in mainland Australia suggests that this effect may have been less severe in Tasmania than mainland Australia.

Biogeographic implications

The low divergence between chloroplast haplotypes observed in *A. moschatum* means that only limited biogeographic inferences can be made for this species. Apart from the northern populations at Monga and the Blue Mountains (discussed below) no parts of the species distribution contained strongly diverged haplotypes or areas of significantly high diversity of related haplotypes indicative of long term occupation. In Tasmania, for example, haplotype H4 an endemic to central western and southern Tasmania has a similar distribution to some haplotypes of both *N. cunninghamii* and *T. lanceolata*, where endemic haplotypes strongly supported the long term occupation by these species. However, the absence of strong divergence of this haplotype (only one nucleotide substitution from H1) means that the possibility that this haplotype evolved in the Holocene cannot be ruled out. In addition, the widespread extent of the ancestral haplotype H1 in Tasmania does not enable understanding the relative contributions of either multiple glacial refugia and/ or rapid Holocene dispersal to its current widespread distribution to be understood. The sharing of one haplotype across nine populations of *Juniperus communis* var. *saxatilis* distributed over a 3000 km area in Europe (Vargas, 2003) was considered as possibly due to the high dispersal ability of the fleshy cones of this species. However, as Chapter 3 indicates caution must be made when predicting the dispersal ability of species based on dispersal traits. The widespread distribution of the H1 haplotype highlights a problem in phylogeography where ancestral haplotypes are found to have a wide distribution and there is insufficient independent evidence available from the fossil record or other sources to interpret this result. In northern Europe, after consideration of extensive fossil records and the known extent of Last Glacial ice, the widespread distributions of ancestral haplotypes of a number of taxa e.g. *Fagus sylvatica* (Demesure *et al.*, 1996), *Frangula alnus* (Hampe *et al.*, 2003), and *Alnus glutinosa* (King & Ferris, 1998) have been interpreted as being the result of a

bottleneck during Holocene expansion, a process described by (Hewitt, 1996), resulting in the subsequent dominance of a single haplotype in formerly glaciated areas. On the other hand, the sharing of an ancestral haplotype between populations of the temperate forest tree *Liquidamber styraciflua* within the main part of its range in the southeastern United States and highly disjunct populations in Mexico and Guatemala has been considered as possibly due to ancient vicariance and subsequent stasis, as supported by fossil evidence for the presence of the species in Mexico from the Miocene (Morris *et al.*, 2008). Similarly the widespread distribution of the H3 haplotype in Victoria and southeastern NSW is uninformative as to the location of glacial refugia, although it must have been derived from and expanded out some time after it split from the ancestral (H1) haplotype, most likely from Tasmania where this ancestral haplotype is endemic. However, the sharing of an endemic haplotype between all *A. moschatum* samples from Victoria and southeastern NSW is of some interest as it provides evidence for the affinity between these populations. This result is in contrast to Ladd (1991) who considered that the *A. moschatum* dominated rainforests of east Gippsland were the 'southern extremity' of cool temperate rainforests that extend discontinuously southwards from the northeast tablelands of NSW and are usually dominated by *Nothofagus moorei* from the Barrington Tops northwards. However, the genetic relationships of the other important rainforest tree in east Gippsland, *Elaeocarpus holopetalus* (Cameron, 1991), have not been investigated.

Some conclusions can also be made of the dispersal of the species. Most regions contained a single haplotype in *A. moschatum* except for western Tasmania. This is in contrast to previous chloroplast phylogeographic studies of some trees with plumose wind dispersed seeds with, for example, strong admixture of six haplotypes observed in ten populations (98 samples) of *Populus tremula* in Italy (Salvini *et al.*, 2001). A similar finding was observed in *Salix caprea* (Palme *et al.*, 2003b) across Europe, with some rare haplotypes having large geographic ranges. Despite a similar dispersal mechanism, *A. moschatum* may differ from these tree species due to its non-pioneer status, and documented poor recruitment meaning that successful establishment of viable populations by long-distance dispersal is relatively rare. However, the widespread distribution of the rare haplotype H2, observed across the eastern half of Tasmania, is consistent with the high effective dispersal ability thought to occur in *A. moschatum*.

The low chloroplast diversity found in *A. moschatum* means that other molecular methods using faster evolving markers, such as nuclear microsatellites or amplified fragment length polymorphisms (AFLPs), may need to be used to help resolve the history of dispersal and isolation of the current populations that are widely geographically separated but share the same chloroplast haplotype. Kropf *et al.*, (2009) describes a technique that allows the estimate of divergence time of current populations using AFLP based data. Nuclear markers are particularly amenable for use in *A .moschatum* due to the fact that the species is insect pollinated and therefore likely to have restricted pollen dispersal (Shapcott, 1995). Alternatively, high throughput DNA sequencing technologies (e.g. Margulies *et al.*, 2005; Meyer *et al.*, 2008) could be utilised to search for more chloroplast variation in *A. moschatum*. In addition, new cpDNA primers that are purported to amplify faster evolving regions of the chloroplast (e.g. Watts *et al.*, 2008) could be investigated.

The northern populations

Both of the most northern populations sampled in this study from Monga NP and the Blue Mountains contained the most diverged haplotypes observed in *A. moschatum*. These populations may be derived from past dispersal events or fragmentation of former wider range and subsequent divergence because they are likely to be derived from a haplotype that is (at least currently) endemic to Tasmania. However, more characters are needed to allow for the application of molecular dating techniques to better understand the timing of these events. Of these northern populations, the most diverged haplotype was found in the Monga National Park, the only population of the species west of Batemans Bay (Floyd, 1990). This finding is significant as it is the first evidence for the distinctiveness of this population and represents a significant component of the chloroplast diversity of the species. These findings, and the fact that the site at present represents the only known association of *A. moschatum* with *Eucryphia moorei* (Mackey *et al.*, 1999), indicate the importance of the protection of this population.

Excluding the possibility of rapid evolution of the chloroplast (a process argued against by the relatively low divergence of *Atherosperma* from its sister genera; see Fig. 2), the divergence of the Monga population indicates the isolation of *A. moschatum* in this region probably through the Last Glacial Maximum. The reason for the isolation of this population is difficult to ascertain. Floyd (1990) considered that

the sensitivity of the species to fire may explain the current isolation of the species to this one, presumably unburnt, gully in the Monga National Park. However, in Tasmania, *A. moschatum* can survive fire and recover by coppicing (Macphail, 1984). Another hypothesis is that the species has been unable to expand its range due to population size effects possibly exacerbated the apparently poor dispersal of *A. moschatum* seed in a closed canopy (Read & Hill, 1988b). Suitable wet and cool conditions for *A. moschatum* presumably exist in the vicinity of the Monga site with cool temperate rainforest dominated by *E. moorei* and *Elaeocarpus holopetalus* occurring extensively at high elevations in Budawang National Park (e.g. at Mt Budawang), including populations less than 5 kilometres away.

The observation of a diverged haplotype in the Blue Mountains is consistent with previous evidence for the long-term isolation of these populations (Schodde, 1969; Shapcott, 1994). However, the significance of this finding cannot be fully understood until the most northern populations in the Barrington Tops region ~ 250 kilometres to the northeast are examined. The Barrington Tops populations are considered the most morphologically diverged populations in the species (Schodde, 1969; Floyd, 1989) and were distinct from the Blue Mountains at the isozyme level (Shapcott, 1994). The occurrence of a unique and diverged haplotype in the Barrington Tops region would support the hypothesis that the northern populations of *A. moschatum* have experienced a similar history of long term isolation. The inclusion of Barrington Tops samples would also resolve the genetic relationships at the chloroplast level of the Blue Mountains and Barrington Tops populations which have been classified as a separate subspecies and better clarify the status of the Monga population.

Appendices

Appendix 1

Atherosperma moschatum sample information for the range-wide chloroplast study (142 samples), including the haplotype of each sample.

N	*Location*	*Region*	*Lat.*	*Long.*	*Alt. (masl)*	*Haplotype*
1	Sunset Ridge	North East Tasmania	-41.367	147.538	924	H1
2	Blue Tier	North East Tasmania	-41.183	148.010	748	H1
3	Blue Tier	North East Tasmania	-41.174	148.011	756	H1
4	Weavers Creek	North East Tasmania	-41.432	147.365	656	H1
5	Mt Albert Rd	North East Tasmania	-41.349	147.967	729	H1
6	Mt Field NP	Western Tasmania	-42.683	146.651	830	H1
7	near Boyd Lookout	Western Tasmania	-42.815	146.359	610	H1
8	Nile River	North East Tasmania	-41.598	147.560	584	H1
9	Nile River	North East Tasmania	-41.597	147.559	585	H1
10	Bennies Creek	North East Tasmania	-41.349	147.389	530	H1
11	St Patricks River	North East Tasmania	-41.293	147.413	437	H1
12	Tatnells Hill	Tasman Peninsula	-43.087	147.954	330	H1
13	Beckett Creek	North East Tasmania	-41.400	147.519	371	H1
14	Snug River	Western Tasmania	-43.085	147.201	205	H1
15	Newall Creek	Western Tasmania	-42.162	145.539	128	H1
16	Bessels Rd	Great Western Tiers	-41.740	146.607	804	H1
17	Meander River	Great Western Tiers	-41.724	146.545	645	H1
18	Trial Harbour Rd	Western Tasmania	-41.899	145.267	294	H1
19	Nelson Falls track	Western Tasmania	-42.104	145.736	380	H1
20	Franklin River	Western Tasmania	-42.216	146.018	448	H1
21	Sandspit Forest Reserve	Weilangta	-42.707	147.842	128	H1
22	Pine Creek	Weilangta	-42.726	147.877	288	H1
23	Lookout Hill Rainforest Ledge	Douglas-Apsley NP	-41.739	148.228	384	H1
24	Lookout Hill Rainforest Ledge	Douglas-Apsley NP	-41.746	148.222	401	H1
25	Meetus Falls, Cygnet River	Eastern Tiers	-41.951	147.885	592	H1
26	Meetus Falls, Cygnet River	Eastern Tiers	-41.953	147.885	496	H1
27	Tom Legges Tier	Eastern Tiers	-42.166	147.882	605	H1
28	Mt Wedge track	Western Tasmania	-42.844	146.284	655	H1
29	Mt Wedge track	Western Tasmania	-42.837	146.279	378	H1
30	Serpentine River	Western Tasmania	-42.777	145.981	199	H1
31	Fergusson Falls	Western Tasmania	-41.903	146.122	810	H1
32	Lake Skinner track	Western Tasmania	-42.945	146.699	705	H1
33	Lake Skinner track	Western Tasmania	-42.945	146.691	830	H1
34	King William Creek, Lyell Hwy	Western Tasmania	-42.212	146.122	791	H1
35	Griffiths Creek, Lyell Hwy	Western Tasmania	-42.211	146.092	756	H1
36	Double Barrel Creek, Lyell Hwy	Western Tasmania	-42.193	145.948	435	H1
37	West Coast Range	Western Tasmania	-41.944	145.555	560	H1
38	Mt Murchison	Western Tasmania	-41.840	145.614	596	H1
39	Snake Creek, Lyell Hwy	Western Tasmania	-42.115	145.786	509	H1
40	trib. of Huskisson River	Western Tasmania	-41.730	145.491	225	H1
41	road to Corrina, nr Pieman River	Western Tasmania	-41.714	145.077	179	H1
42	Lefroy Ridge, nr Pieman River	Western Tasmania	-41.678	145.080	199	H1
43	The Longback, nr Donaldson River	Western Tasmania	-41.564	145.088	295	H1
44	Donaldson River	Western Tasmania	-41.479	145.103	259	H1
45	nr Leigh River	Western Tasmania	-41.363	145.031	269	H1
46	nr Edith Creek	Western Tasmania	-41.136	144.951	81	H1
47	Arthur River	Western Tasmania	-41.115	144.984	61	H1
48	Harry Ryan Creek	Western Tasmania	-41.043	145.124	182	H1
49	Peegra Rd	Western Tasmania	-41.052	145.312	293	H1
50	McDougalls Hill	Western Tasmania	-43.577	146.888	12	H1
51	Hot Springs Creek, nr Hastings Cave	Western Tasmania	-43.391	146.850	94	H1
52	Hastings Cave	Western Tasmania	-43.385	146.841	136	H1
53	Chestermans Road	Western Tasmania	-43.391	146.858	145	H1
54	Chestermans Road	Western Tasmania	-43.389	146.865	238	H1
55	Creekton Rivulet	Western Tasmania	-43.375	146.893	107	H1
56	Ferrars Tier nr Lake Leake	Eastern Tiers	-41.999	147.859	690	H1
57	Cygnet River	Eastern Tiers	-41.944	147.873	650	H1

58	Cygnet River- upper reaches	Eastern Tiers	-41.929	147.848	785	H1
59	Big Sassy Creek	Eastern Tiers	-42.152	147.899	482	H1
60	Dennistoun Road	Western Tasmania	-42.257	147.102	660	H1
61	Ironbound Range East	Western Tasmania	-43.513	146.467	715	H1
62	Ironbound Range East	Western Tasmania	-43.513	146.467	715	H1
63	Louisa Beach	Western Tasmania	-43.514	146.360	43	H1
64	Louisa Beach	Western Tasmania	-43.514	146.360	43	H1
65	Quamby Bluff	Great Western Tiers	-41.658	146.700	925	H1
66	Howell Gorge	Dazzler Ranges	-41.276	146.767	290	H1
67	Howell Falls	Dazzler Ranges	-41.259	146.773	205	H1
68	Saxons Creek	Dazzler Ranges	-41.270	146.702	143	H1
69	Bishop and Clerk	Maria Island	-42.592	148.114	550	H1
70	Fraser River	King Island	-39.930	144.019	82	H1
71	Fraser River	King Island	-39.915	144.019	80	H1
72	Cathedral Rock	Western Tasmania	-42.940	147.192	740	H1
73	Mt Strzelecki	Flinders Island	-40.201	148.072	720	H1
74	Endeavour Bay	Western Tasmania	-42.651	145.347	18	H1
75	South Lune Road	Western Tasmania	-43.464	146.858	82	H1
76	Angel Rain, Franklin River	Western Tasmania	-42.218	145.906	316	H1
77	Finchams Crossing, Franklin River	Western Tasmania	-42.242	145.767	219	H1
78	Camp Arcade, Franklin River	Western Tasmania	-42.285	145.747	231	H1
79	Coruscades, Franklin River	Western Tasmania	-42.329	145.793	181	H1
80	Rafters Basin, Franklin River	Western Tasmania	-42.365	145.772	113	H1
81	Newlands Cascade, Franklin River	Western Tasmania	-42.422	145.756	124	H1
82	Blackmans Bend, Franklin River	Western Tasmania	-42.517	145.768	26	H1
83	Sir John Falls	Western Tasmania	-42.570	145.690	21	H1
84	Whalers Cove	Western Tasmania	-43.269	145.925	7	H1
85	Liffey Falls State Reserve	Western Tasmania	-41.692	146.758	650	H1
86	Liffey Falls State Reserve-Liffey River	Western Tasmania	-41.701	146.758	520	H1
87	Liffey Falls State Reserve	Western Tasmania	-41.701	146.764	580	H1
88	tributory of Black Rivulet	North East Tasmania	-41.192	147.792	420	H2
89	NW tributory of Patersonia Rivulet	North East Tasmania	-41.291	147.319	448	H2
90	Liffey Falls State Reserve	Western Tasmania	-41.690	146.760	668	H2
91	Forestier Peninsula	Forestier Peninsula	-42.942	147.915	231	H2
92	western side of Mt Latrobe	Wilsons Promontory, Vic.	-39.001	146.372	640	H3
93	western side of Mt Latrobe	Wilsons Promontory, Vic.	-39.001	146.372	649	H3
94	Tarra Bulga NP, Macks Creek	Strzelecki Ranges, Victoria	-38.427	146.569	589	H3
95	Tarra Bulga NP, Macks Creek	Strzelecki Ranges, Victoria	-38.427	146.569	594	H3
96	headwaters of Hope Creek, East Tanjil Rd	Central Highlands, Victoria	-37.849	146.253	1218	H3
97	headwaters of Hope Creek, East Tanjil Rd	Central Highlands, Victoria	-37.846	146.247	1141	H3
98	Myrrhee	Central Highlands, Victoria	-37.782	146.138	1104	H3
99	Myrrhee	Central Highlands, Victoria	-37.775	146.141	1086	H3
100	Ythan Creek	Central Highlands, Victoria	-37.722	145.683	1051	H3
101	Cement Creek East Branch	Central Highlands, Victoria	-37.700	145.718	811	H3
102	Archeron River, nr Archeron Gap	Central Highlands, Victoria	-37.669	145.741	796	H3
103	Boobyalla Saddle	Central Highlands, Victoria	-37.687	145.716	819	H3
104	Cameron Cascade	Central Highlands, Victoria	-37.527	145.850	1085	H3
105	Snowy Junction, Cumberland Road	Central Highlands, Victoria	-37.534	145.835	1008	H3
106	Archeron Way, nr Somers Park	Central Highlands, Victoria	-37.636	145.713	575	H3
107	Bonang River, Errinundra Plateau	East Gippsland	-37.209	148.738	706	H3
108	Bonang River, Errinundra Plateau	East Gippsland,	-37.213	148.742	705	H3
109	Result Creek, Errinundra Plateau	East Gippsland	-37.248	148.786	883	H3
110	Result Creek, Errinundra Plateau	East Gippsland	-37.243	148.792	905	H3
111	Clarksville Rd, Errinundra Plateau	East Gippsland	-37.235	148.889	1027	H3
112	Goonmirk Rocks, Errinundra Plateau	East Gippsland	-37.276	148.889	1199	H3
113	Rutherford Creek	Brown Mountain, NSW	-36.595	149.442	843	H3
114	Rutherford Creek	Brown Mountain, NSW	-36.595	149.441	841	H3
115	Sherbrooke	Dandenong Ranges, Vic.	-37.886	145.368	352	H3
116	Sherbrooke	Dandenong Ranges, Vic	-37.887	145.368	348	H3
117	tributory of Leather Barrel Creek	Kosciusko NP, NSW	-36.532	148.198	1150	H3
118	Twins Creek, tributory of Geehi River	Kosciusko NP, NSW	-36.323	148.278	1040	H3
119	tributory of Leather Barrel Creek	Kosciusko NP, NSW	-36.532	148.198	1150	H3
120	tributory of Leather Barrel Creek	Kosciusko NP, NSW	-36.532	148.198	1150	H3
121	near Lake Gordon	Western Tasmania	-42.811	146.272	338	H4
122	Florentine Road	Western Tasmania	-42.718	146.508	482	H4
123	Creepy Crawly	Western Tasmania	-42.832	146.385	589	H4
124	Lake Dobson	Western Tasmania	-42.683	146.590	1034	H4
125	Tatnells Hill	Tasman Peninsula	-43.084	147.925	455	H4

126	Mount Mangana	Bruny Island	-43.361	147.287	450	H4
127	Wylds Craig eastern slope	Western Tasmania	-42.470	146.408	910	H4
128	Wylds Craig track	Western Tasmania	-42.491	146.434	605	H4
129	Russel Falls. Mt Field NP	Western Tasmania	-42.680	146.712	180	H4
130	Cockle Creek	Western Tasmania	-43.580	146.899	2	H4
131	Catamaran River	Western Tasmania	-43.558	146.889	14	H4
132	Griffiths Creek, Lyell Hwy	Western Tasmania	-42.213	146.092	779	H4
133	Christmas Cove	Western Tasmania	-42.724	145.392	20	H4
134	Cowrie Beach	Western Tasmania	-42.972	145.558	22	H4
135	Mulcahy River	Western Tasmania	-43.107	145.716	22	H4
136	Gorilla Ridge	Western Tasmania	-43.289	146.451	620	H4
137	Penance Grove	Monga NP	-35.601	149.913	707	H5
138	Penance Grove	Monga NP	-35.601	149.913	705	H5
139	Penance Grove	Monga NP	-35.601	149.913	717	H5
140	Bonnie Doon Falls, Nellies Glen	Blue Mountains	-33.710	150.290	820	H6
141	Leura Falls (lower)	Blue Mountains	-33.723	150.320	750	H6
142	Bonnie Doon Falls, Nellies Glen	Blue Mountains	-33.710	150.290	820	H6

Appendix 2

Genbank accession numbers for outgroups. Blank spaces indicate missing sequences for *D. tenuipes*.

Fragment	*L. sempervirens*	*N . viellardii*	*D. tenuipes*
K1–*mat*K1	GQ302625	GQ302626	
*mat*K6–K2	GQ302627	GQ302628	GQ302629
*pet*N1–*psb*M f	GQ302632	GQ302633	
*pet*N1–*psb*M r	GQ302630	GQ302631	
*trn*T–*trn*L	GQ302619	GQ302620	GQ302621
*trn*L intron	GQ302622	GQ302623	GQ302624

Chapter 5: Preliminary investigation of chloroplast variation in three Tasmanian endemic cool temperate rainforest plants: *Olearia persoonioides* (Asteraceae), *Phyllocladus aspleniifolius* (Podocarpaceae) and *Telopea truncata* (Proteaceae).

Introduction

Few cool temperate rainforest woody plants endemic to Tasmania occur in the drier eastern half of the island. Genetic evidence from previous chapters have provided strong evidence that two cool temperate rainforest species, *Nothofagus cunninghamii* (Chapter 2) and *Tasmannia lanceolata* (Chapter 3) survived in parts of eastern Tasmania during the last glacial and possibly for multiple glacial cycles. However, a chloroplast phylogeographic study of another species, *Atherosperma moschatum*, that is widespread in eastern Tasmania, was not able to discern between the competing hypothesis of Holocene dispersal or glacial survival for its occurrence in eastern Tasmania (Chapter 4). Whether eastern Tasmania also harboured glacial refugia for other cool temperate rainforest species that currently occur in eastern Tasmania is of particular interest considering the inhospitable dry climates that are considered to have occurred in eastern Tasmania during the last glacial. This chapter describes a preliminary investigation into the chloroplast variation using DNA sequencing and PCR/RFLP of three species of woody plants that are widespread in western Tasmania and have disjunct population in eastern Tasmania, the shrub *Olearia persoonioides* (Asteraceae), the conifer *Phyllocladus aspleniifolius* (Podocarpaceae) and the shrub *Telopea truncata* (Proteaceae). Due to the low variation observed in these species this chapter gives an abbreviated treatment of the methods and results.

Materials and Methods

Study species

Olearia persoonioides (DC.) Benth. (Asteraceae)

Olearia persoonioides is a shrub 1 to 3m high endemic to Tasmania (Curtis & Morris, 1994). As in other members of the genus, *O. persoonioides* produces a single seeded dry indehiscent fruit (an achene) with a pappus that aids in the wind dispersal of the

seed. The species is widely distributed in the western half of Tasmania (Fig. 1a) where it is a common component of cool temperate rainforests (Jarman & Brown, 1983) from near sea-level to ~ 1000 masl, but is most common in montane areas. The species can also be found in vegetation types other than cool temperate rainforest such as wet sclerophyll forest, subalpine *Eucalyptus* forest and *Melaleuca* and *Leptospermum* dominated shrubbery (Balmer & Corbett, 2001). The species has one very isolated occurrence in the northeast highlands of Tasmania at Mount Michael in the Blue Tier area (Fig. 1a) where it occurs within thamnic cool temperate rainforest and subalpine shrubland.

Phyllocladus aspleniifolius Labill.) Hook. f (Podocarpaceae)

Phyllocladus aspleniifolius (celery-top pine) is a conifer up to 30 m in height that is endemic to Tasmania. The species is usually dioecious and produces seed with a fleshy aril (Curtis & Morris, 1994) that are dispersed by birds (Kirkpatrick, 1977; Barker & Kirkpatrick, 1994), although the majority probably fall near the parent plant (Read, 1999). The species is widespread in the western half of Tasmania but also is found in disjunct populations in the northeast highlands and in isolated patches in southeastern Tasmania in the Weilangta area and Maria Island (Neyland & Brown, 1993) (Fig. 1b). *Phyllocladus aspleniifolius* occurs from sea-level to 1200 masl and is a common tree of cool temperate rainforests and wet eucalypt forest (Barker, 1995) and can occur in shrub from above the climatic tree line. The species usually co-occurs with other cool temperate rainforest trees but may dominate some sites on acidic and infertile soils (Jackson, 1983). Historical records indicate that it was present on King Island at the time of European settlement ~200 years ago (Jennings, 1959).

Telopea truncata (Labill.) R. Br. (Proteaceae)

Telopea truncata (waratah) is a shrub or small tree up to 8 m tall (Curtis & Morris, 1994). The species is monoecious with hermaphroditic flowers. The fruit is a woody, dehiscent follicle (Curtis & Morris, 1994) that contains seeds with a terminal wing that are considered to be poorly wind dispersed (Read & Hill, 1983). *Telopea truncata* is one of five species in the genus (McCarthy, 1995) that is confined to southeastern Australia but is the only species found in Tasmania where it is endemic. The species is widespread from sea-level to 1200 masl in western Tasmania but is confined to higher altitudes above ~ 500 masl across the northeast highlands (Fig. 1c).

Telopea truncata is commonly recorded in the understorey of cool temperate rainforest (Jarman & Brown, 1983), particularly at high altitudes, and is also is a common component of subalpine shrubberies (Curtis & Morris, 1994).

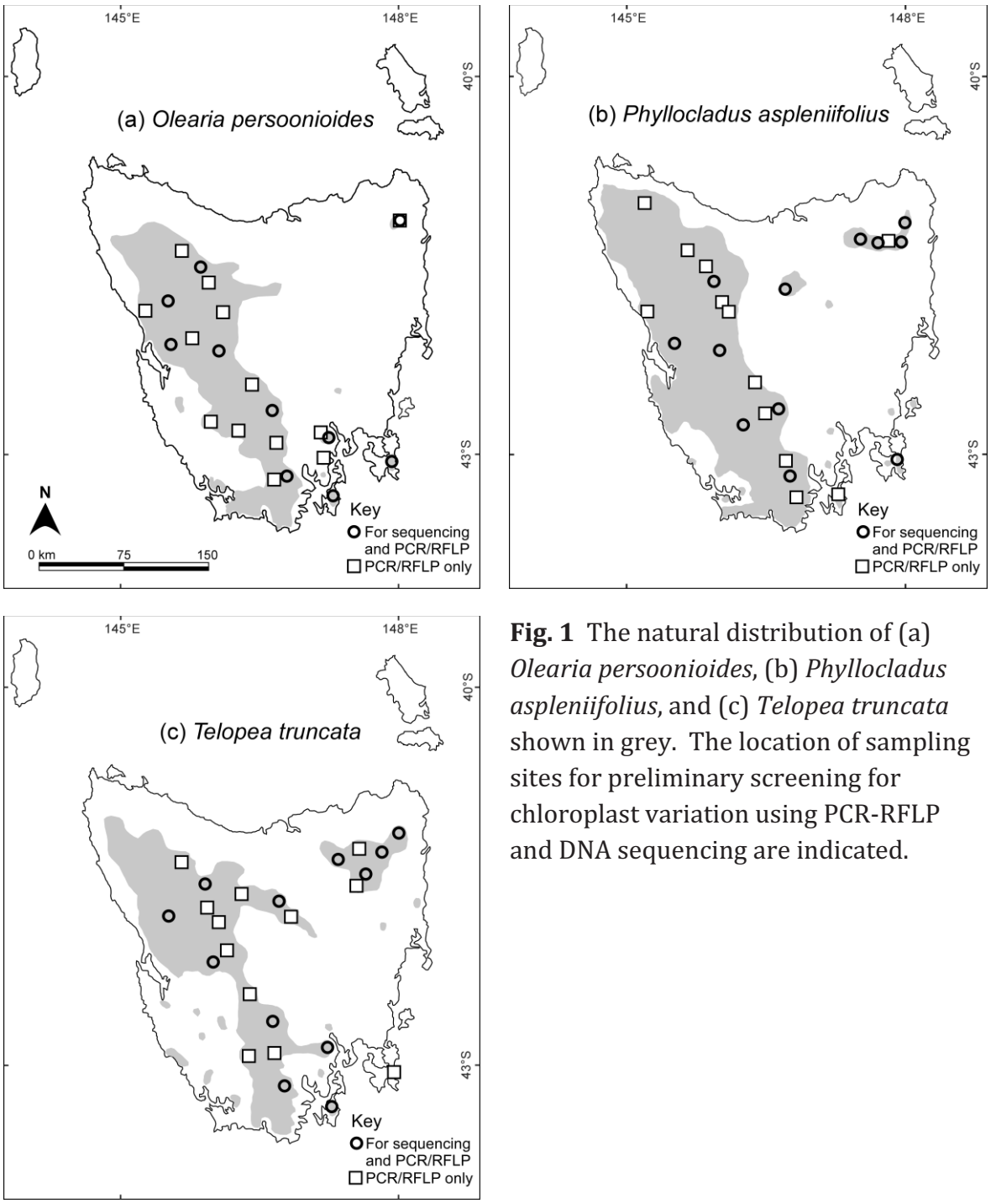


Fig. 1 The natural distribution of (a) *Olearia persoonioides*, (b) *Phyllocladus aspleniifolius*, and (c) *Telopea truncata* shown in grey. The location of sampling sites for preliminary screening for chloroplast variation using PCR-RFLP and DNA sequencing are indicated.

Sampling

A total of 24 samples of *O. persoonioides*, *P. aspleniifolius* and *T. truncata* with the objective to obtain one sample per site and collect from most major parts of their current ranges in western and eastern Tasmania. Three samples of *O. persoonioides* were collected from its only known occurrence in eastern Tasmania at the Blue Tier.

Molecular Methods

Two PCR based methods, PCR/RFLP and DNA sequencing, were used to screen for chloroplast variation within the three species. All 24 samples collected per species were included in the PCR/RFLP survey, while half of these samples were included in a screening using DNA sequencing of a different set of chloroplast primer pairs. DNA extractions, PCR/RFLP and DNA sequencing techniques used were the same as in Chapter 4. Chloroplast primer pairs used were also the same for those used in chapter 4 except for the fragments *atp*I–*rpo*C2 (IC2) and *rpo*C2-f–*rpo*C2-r (C2C2) (Grivet *et al.*, 2001) and an additional primer pair used for DNA sequencing *trn*V–*ndh*C (Shaw *et al.*, 2007). The abbreviations of primer pairs used are the same as those in Chapter 4.

Results and discussion

Olearia persoonioides- PCR/RFLP

In total eight primer pairs of the 17 tried produced single banded and high yielding fragments totalling 16,300 bp that were suitable for PCR-RFLP analysis (Table 1). Of the 24 fragment/ restriction endonuclease combinations two polymorphisms were identified (Table 2).

Table 1 Fragments that were successfully amplified in *O. persoonioides, P. aspleniifolius and T. truncata* for PCR-RFLP are indicated by the approximate sizes in base pairs of the amplified fragments.

Fragment	*O. persoonioides*	*P. aspleniifolius*	*T. truncata*
AE	1500	–	–
L106	–	–	–
pB	–	–	2800
HI	1200	900	1400
OA	–	–	3000
B2B3	–	–	–
DT	–	1500	–
BD	1700	–	1500
SG	–	–	1250
K2Q	–	3200	–
AS	2800	–	2800
CS	–	–	–
ST	1400	–	1400
IC2	2100	2300	2100
C2C2	3200	2900	3200
HK	2400	–	2450
Sf_M	–	1500	–

Table 2 The number of restriction sites after digestion by the three restriction endonucleases in the subset 24 samples of *Olearia persoonioides*. Number of restriction sites per 'normal' sample. The fragment/ restriction endonuclease combinations that were successful in detecting variation are shaded grey.

Fragment	*Taq*I	*Hinf*I	*Hae*III
AE	2	4	1
HI	5	3	0
BD	2	3	1
AS	7†	6	4†
ST	1‡	3‡	2‡
IC2	4	5	4
C2C2	5	6	4
HK	0	2	1

† denotes that the same polymorphism is inferred as having been detected by both *TaqI* and *HaeIII*. ‡ denotes that the same polymorphism is inferred as having been detected by *TaqI*, *HinfI* and *HaeIII*.

An indel of ~ 20 bp in length was detected in the AS fragment by both *Taq*I and *Hae*III. This indel was detected only in two samples from the northwest of Tasmania. Another polymorphism was detected in the ST fragment by all three restriction endonucleases. This polymorphism was detected in five samples from western and southern Tasmania. The distribution of the three *O. persoonioides* haplotypes that are defined by these two indels is shown in Fig. 2.

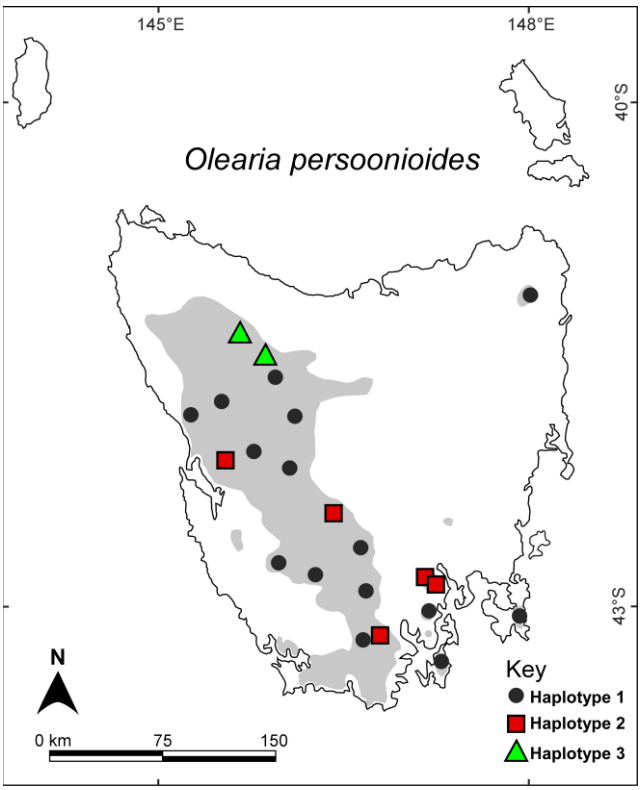


Fig. 2 Distribution of the three haplotypes detected in the 24 samples of *Olearia persoonioides* by PCR-RFLP, with the distribution of the species shown (grey area).

Olearia persoonioides- DNA sequencing

Of the nine primer pairs tested seven produced a single banded product suitable for DNA sequencing (Table 3). In total 4,203 bp of aligned sequence was obtained for the 12 samples. The aligned sequence lengths for each fragment are as follows: *atp*B-*rbc*L 437 bp (GenBank accession number FJ860959); *mat*K6–K2 807 bp (FJ860960); *pet*N–*psb*M 621 bp (FJ860961); *psb*M–*trn*D 675-676 bp (two variants, FJ860962 and FJ860963); *trn*V–*ndh*C F 562 (FJ860964); *trn*V–*ndh*C R 243-245 bp (FJ860965 and FJ860966); *trn*L intron 464 bp (FJ860967); and *trn*L–*trn*F 391 bp (FJ860968). One SSR polymorphism was observed in one sample from Mt Michael in the *psb*M–*trn*D

fragment and another SSR in the *trn*V–*ndh*C fragment observed in one sample from northern Tasmania (results not shown).

Table 3 Primer pairs that were successfully amplified in *Olearia persoonioides* for DNA sequencing are indicated by the approximate sizes in base pairs of the fragments.

Fragment	*O. persoonioides*	*P. aspleniifolius*	*T. truncata*
atpB–rbcL	900	–	1000
K1–matK1	–	900	950
matK6–K2	900	700	700
petN1–psbM	700	–	1500
psbM–trnD	750	–	1200
trnV–ndhC	800	1000	650
trnT–trnL	–	450	700
trnL intron	600	550	650
trnL–trnF	400	450	350

Phyllocladus aspleniifolius-PCR/RFLP

In total six primer pairs of the 17 tried produced single banded and high yielding fragments totalling 12,300 bp that were suitable for PCR-RFLP analysis (Table 1). Of the 19 fragment/ restriction endonuclease combinations, one ~ 30 bp indel was detected among the 24 samples with the C2C2/ *Taq*I and C2C2/ *Hinf*I combinations (Table 4). This indel was observed in one sample from Projection Bluff in northern Tasmania. This polymorphism was subsequently not detected in any of six individuals collected from this region, including one sample from the same stand (results not shown).

Table 4 The number of restriction sites after digestion four by restriction endonucleases in the 24 samples of *Phyllocladus aspleniifolius*. No variation (SNP's or indel's) were detected.

Fragment	*Taq*I	*Hinf*I	*Hae*III	*Ssp*I
HI	2	3	1	–
DT	1	3	2	–
K2Q	4	6	2	–
IC2	4	4	3	–
C2C2	5†	4†	1	–
Sf_M	3	1	0	2

† denotes that the same polymorphism is inferred as having been detected by the restriction endonucleases *Taq*I and *Hinf*I.

Phyllocladus aspleniifolius-DNA sequencing

Of the nine primer pairs tested six produced a single banded product suitable for DNA sequencing (Table 3). In total 2,024 bp of aligned sequence was obtained for the 12 samples. The aligned sequence lengths for each fragment are as follows: *mat*K6–K2 were 784 bp (GenBank accession number FJ860947), for the *trn*T–*trn*L intergenic spacer 418 bp (FJ860948), *trn*L intron 443 bp (FJ860949) and the *trn*L–*trn*F intergenic spacer 379 bp (FJ860950). The sequencing of K1–*mat*K1 and *trn*V–*ndh*C fragments failed. No SNP, indel or SSR variation was observed in the 12 samples of *P. aspleniifolius*.

Telopea truncata-PCR-RFLP

In total ten primer pairs of the 17 tried produced single banded and high yielding fragments totalling 21,900 bp that were suitable for PCR-RFLP analysis (Table 1). No polymorphisms were detected between the 24 samples using a total of 27 restriction endonuclease/ fragment combinations (Table 5).

Table 5 The number of restriction sites after digestion by three restriction endonucleases in the 24 samples of *Telopea truncata*. No variation (SNP or indel) were detected.

Fragment	*Taq*I	*Hinf*I	*Hae*III
pB	6	3	3
HI	4	3	1
OA	5	7	2
BD	3	5	3
AS	6	4	3
ST	1	2	1
IC2	9	5	4
C2C2	6	6	–
HK	0	2	1

Telopea truncata-DNA sequencing

All nine primer pairs tested produced a single banded product suitable for DNA sequencing (Table 3). In total 5,492 bp of aligned sequence was obtained for the 12 samples. The aligned sequence lengths for each fragment are as follows: *atp*B–*rbc*L 827 bp (FJ860938), K1-*mat*K1 600 bp (FJ860939), *mat*K6–K2 780 bp (FJ860940), *pet*N–*psb*M 829 bp (FJ860941), *psb*M–*trn*D 840 bp (FJ860942), *trn*V–*ndh*C 574 bp (FJ860943), *trn*T–*trn*L 260 bp (FJ860944), *trn*L intron 514 bp (FJ860945) and *trn*L–

*trn*F 268 bp (FJ860946). No SNP, indel or SSR variation was observed in the 12 samples of *T. truncata*.

Chapter 6: Conclusions

Outline of discussion

By investigating the genetic patterns of the chloroplast of three widespread plants of cool temperate rainforest in southeastern Australia, *Nothofagus cunninghamii*, *Tasmannia lanceolata* and *Atherosperma moschatum*, this thesis has enabled a new understanding of how these species have responded to past climatic changes during the Pleistocene. Because of their relatively high chloroplast diversity, it was possible to make stronger inferences concerning the history of *N. cunninghamii* and *T. lanceolata* than for *A. moschatum*, which showed low chloroplast diversity. As a result this discussion will focus mainly on *N. cunninghamii* and *T. lanceolata*. The major themes that will be discussed are the evidence for the importance of tolerance or adaptation of these species during past climatic changes, the limited mobility in *N. cunninghamii* and *T. lanceolata* inferred from the patterns of the seed-mediated chloroplast and how this limited dispersal contrasts with the northern hemisphere, and the depth of phylogeographic patterns in the species that reflect events that occurred long before the last glacial.

The roles of adaptation and tolerance in the history of southern cool temperate rainforests

A principal finding of this thesis is the strong genetic signal for multiple glacial refugia in both *Nothofagus cunninghamii* and *Tasmannia lanceolata*. Considering that survival within refugia is unlikely to always result in genetic patterns that signal the presence of refugia, the number of refugia is likely to be greater than can be inferred from the chloroplast patterns. For example, different refugial populations of a species may share the same haplotype. In addition, although chloroplast patterns may indicate survival of one species in an area the genetic signal may be ambiguous for other plants in the same area. For example, presence of an endemic and diverged haplotype of *N. cunninghamii* in northeast Tasmania contrasts with the occurrence of a single widespread haplotype in *T. lanceolata* in the same region.

Evidence for multiple glacial refugia fits well with expectations for the southern hemisphere that species were able to survive during glacials in numerous topographically protected sites (Macphail & Colhoun, 1985; McGlone, 1985; Markgraf *et al.*, 1995). Importantly, this study provides strong evidence that locations of survival for forest species included areas that would be unexpected based on our

current understanding of paleoclimates and the climatic tolerances of species inferred from the current climatic ranges. The strongest cases for this are evidence for possible multiple refugia for *N. cunninghamii* in northeast Tasmania, the evidence for survival of this species above the LGM climate tree line in western Tasmania and genetic evidence that *T. lanceolata* withstood the predicted dry glacial climates in southeastern Tasmania and the Grampians in Victoria. Both southeastern Tasmania and the Grampians are currently at the lower end of rainfall for the species (i.e. 800-1000mm per annum), and there is evidence that, like northeast Tasmania (see Chapter 2), southeastern Tasmania was strongly affected by glacial aridity during the last glaciation (Colhoun, 1977,1985b, 2002). Within each of these regions the presence of divergent, endemic chloroplast lineages are strong evidence for refugial status. However, each of these regions shows little diversity, a pattern entirely consistent with the strong bottlenecks that would be expected in dry climates for rainforest species. This inference is supported by the higher chloroplast diversity observed in western Tasmania where glacial climates are thought to have been much wetter then the east. These findings contribute to a growing understanding of the ability of populations of some woody plants to withstand extreme climates for thousands of years and the importance of the resilience of plants in shaping the current biota (Petit *et al.*, 2008).

The genetic evidence for the survival of *N. cunninghamii* and *T. lanceolata* in apparently hostile climates raises some questions about how these species were able to adapt or tolerate past climatic change. One hypothesis is that both *N. cunninghamii* and *T. lanceolata* were able to tolerate glacial climates in non-analogue environments, that is, combinations of climate and environmental factors that no longer exist in the present interglacial. This proposal is based on the idea that the distribution of these species in the current interglacial may not reflect the full breadth of the ecological range of these species during different parts of the Pleistocene. Another alternative is adaptation where the species were able to survive glacial climates via changes in the functional traits of these species. Changes in functional traits, and therefore the potential climatic range of the species, may have been achieved through plasticity or the evolution of new gene variants. At least during the last glacial the acquisition of genes via hybridisation with other species was impossible in *N. cunninghamii*, *A. moschatum* and (within Tasmania at least) in *T. lanceolata* unless they co-existed with unknown extinct species at the time.

Limited mobility during the Holocene

A strong feature of the chloroplast phylogeographic patterns of both *N. cunninghamii* and *T. lanceolata* is the evidence for very limited mobility. The geographical extent of haplotypes and the low mixing of haplotypes, particularly in *T. lanceolata* indicates that migration of these species out of glacial refugia within their current ranges during the Holocene probably occurred over distances less than 100 km, and in many cases the distance can be inferred to be less than 50 km. The low chloroplast divergence observed between most haplotypes in *A. moschatum* and the widespread occurrence of an ancestral haplotype means that it is difficult to infer movements in this species. The low cpDNA diversity observed in *A. moschatum* compared to both *N. cunninghamii* and *T. lanceolata* may be due to stronger bottlenecks, and means that the current wide range of this species may be explained by the capacity for long-distance seed dispersal of this wind dispersed species. However, this assertion is tentative and awaits further investigation via faster evolving genetic markers (see Chapter 4).

The evidence for limited mobility in the Holocene of *N. cunninghamii* and *T. lanceolata* contrasts with the northern hemisphere where, despite the increasing recognition of small northern glacial refugia that occurred close to the ice sheets (McLachlan *et al.*, 2005; Anderson *et al.*, 2006; Petit *et al.*, 2008), migrations extending over 1000's of kilometres must be inferred to account for the spread of species into formerly glaciated landscapes. Some possible explanations can be forwarded here for the discrepancy between the low mobility that seems to characterise the cool temperate rainforest of southeastern Australia and the significant mobility inferred for many forest trees during the Holocene in the northern hemisphere. Firstly, unlike in the northern hemisphere, expansive areas of more or less continuous and suitable habitat did not become available in southeastern Australia at the end of the Holocene. The patchy distribution of available habitat in southeastern Australia broken by drier landscapes unsuitable for rainforest would have decreased the chance of long-distance dispersal events reaching suitable mesic sites. In addition within the mesic pockets that were made available during the Holocene for rainforest plants, species recovery from glacial climates was dominated by expansion from local refugia. As described by Hewitt (1996) initial founding populations increase exponentially and subsequent colonization events are less likely to be successful after the community dynamics changes to replacement dynamics in more closed vegetation at carrying

capacity. Another contributing factor may be the landscape differences between southeastern Australia and Europe and North America lead to selection for intrinsically higher dispersability in parts of the north compared to the south(i.e. selection of tolerant, 'stay at home' species in the south versus highl mobility in parts of species ranges in the north). For example, phenotypic differences have been observed between populations of *Frangula alnus* in formerly glaciated northern Europe and Mediterranean populations (faster generation time and smaller fruits more suited to migratory birds in northern populations; Hampe & Bairlein, 2000). A similar case has been observed in northern European populations of *Pinus contorta* which reportedly have more mobile wind-dispersed seeds than southern European populations (Cwynar & MacDonald, 1987). The chloroplast phylogeographies of southeastern Australia most closely resemble the phylogeographies of temperate forest species at similar latitudes in the Mediterranean mountains of southern Europe with evidence for low mobility and deep divergence between current populations (Petit *et al.*, 2005).

Antiquity of haplotypes and geographic patterns of haplotypes

The deep divergence of haplotypes observed between some populations across the ranges of *N. cunninghamii* and *T. lanceolata* provides strong evidence that the current chloroplast structure of these species reflects events that occurred long before the last glacial. This contributes to a growing number of studies of temperate plants that have observed deep phylogeographic patterns, for example, in the Mediterranean region (Lumaret *et al.*, 2002; Petit *et al.*, 2005; Magri *et al.*, 2007), California (Grivet *et al.*, 2006) and southeastern United States (Morris *et al.*, 2008). The nesting of *N. moorei* within the chloroplast variation observed in *N. cunninghamii* provides evidence for great depth of divergence of lineages within *N. cunninghamii* (see Chapter 2). Though the complete relationship between postglacial populations and their modern genetic diversity cannot be fully understood without the analysis of DNA haplotypes from fossil plants (Magri *et al.*, 2006), the chloroplast phylogeography of *N. cunninghamii* provides evidence of stasis of the species with a highly diverged lineage restricted to the northeast highlands of Tasmania and multiple lineages in western Tasmania. Though a temporal element (Morris *et al.*, 2008) is absent in *T. lanceolata* and *A. moschatum* (e.g. molecular dating), apart from some evidence for the slow evolution of the chloroplast, the strong divergence of endemic haplotypes or lineages in some regions within the ranges of these species is

consistent with long term occupation of areas possibly for multiple glacials, for example, in southeastern Tasmania, the Grampians, and western Tasmania for *T. lanceolata* and Monga and possibly the Blue Mountains in *A. moschatum.* Whether the morphological similarity that is observed between most of these populations that have probably been isolated for multiple glacials is a result of morphological stasis (e.g. Dick *et al.*, 2003), consistent with the slow evolution of trees (Petit & Hampe, 2006), or pollen mediated gene flow between these regions needs to be examined.

Although some populations of *N. cunninghamii* and *T. lanceolata* may have remained isolated for long periods, the shallow divergence (i.e. one or two mutations) of haplotypes observed between populations of these species in parts of Tasmania and the Central Highlands of Victoria (and the Australian Alps for *T. lanceolata*) provides evidence that both species must have been able to migrate across the 250 km wide Bass Strait. Considering the fact that populations of both species in the Central Highlands of Victoria harbour endemic haplotype clades that are nested within the chloroplast diversity present in Tasmania, any migration is most likely to have occurred before the last glacial and the direction of movement is most easily explained as having occurred northwards from Tasmania. A similar argument could be made for *A. moschatum*, although because the single haplotype of this species in Victoria and southeastern NSW differs from Tasmania by only one mutation a Holocene dispersal cannot be ruled out. Both *N. cunninghamii* and *T. lanceolata* may have been able to migrate during a similar event such as the convergence of suitable climates and the opening of Bass Strait which has been exposed repeatedly throughout the Pleistocene, as has been postulated for *N. cunninghamii* (Howard & Hope, 1970; Bridgewater, 1976; Hope, 1994). The genetic evidence for pre-last glacial migration of both *N. cunninghamii* and *T. lanceolata* across Bass Strait fits well with chloroplast phylogeographic studies of *Eucalyptus* species that occur in both Tasmania and southern Victoria including *E. globulus* (Freeman *et al.*, 2001), *E. viminalis, E. ovata* (Marthick, 2005) and *E. regnans* (Nevill et al., 2008).

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